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(71) Applicant: pION, INC. [US/US]; 5 Constitution Way, Woburn, MA 01801 (US).

(72) Inventors: AVDEEF, Alex; Apt. 9, 261 Commonwealth Ave., Boston, MA 02116 (US). TSINMAN, Konstantin, L.; 255 North Road, Unit 132, Chelmsford, MA 01824 (US).

(74) Agents: GAGNEBIN, Charles, L., III et al.; Weingarten, Schurgin, Gagnebin & Hayes, LLP, Ten Post Office Square, Boston, MA 02109 (US).

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(54) Title: MEASUREMENT OF SOLUBILITY-pH PROFILES

(57) Abstract: This invention relates to a method and an analytical device for the determination of solubility of a compound. The basic method involves determinig solubility of a compound by measuring the UV spectrum of a reference solution of the compound, under conditions avoiding or suppressing precipitation, and comparing it to the UV spectrum of a saturated sample solution of the compound. Variations of the basic method include: (a) making reference solutions either by dilution of the sample solution to the point where precipitation is avoided, or by adding a water-miscible cosolvent to the sample solution so that precipitation is suppressed, and comparing the UV absorbances of the compound under reference conditions to the compound in a saturated solution, (b) determining the true aqueous solubility from the effect on the pK_a that results from dissolving the compound in an aqueous solution containing some DMSO (typically 0.1 - 5% v/v), and (c) correcting concentrations determined from the UV absorbance values for impurities.

TITLE OF THE INVENTION

MEASUREMENT OF SOLUBILITY-pH PROFILES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 60/178,616 filed January 28, 2000, which is incorporated in its entirety herein.

FIELD OF THE INVENTION

The measurement of aqueous solubility in a high-throughput screening environment plays an important role in the selection of the most promising drug candidate molecules in pharmaceutical research and development. The invention described involves a method, a simple, robust, high-throughput screen, that is applicable for the determination of the equilibrium solubility of sparingly soluble compounds and that may be used in pharmaceutical, biotechnology, and related industries. An analytical device has been designed to implement this solubility measurement technique.

BACKGROUND OF THE INVENTION

The 1990 treatise by Grant and Higuchi [D.J.W. Grant, T. Higuchi, Solubility Behavior of Organic Compounds, John Wiley & Sons: New York, 1990] comprehensively covers the known art. Many protocols have been described in the literature [S. Venkatesh, J. Li, Y. Xu, R. Vishnuvajjala, B.D. Anderson, *Pharm. Res.* **1996**,*13*, 1453-1459; A. Avdeef, *Pharm. Pharmacol. Commun.*

1998, *4*, 165-178; A. Avdeef, C.M. Berger, C. Brownell, *Pharm. Res.* **2000**, *17*, 85-89; A. Avdeef in B. Testa, H. van de Waterbeemd, G. Folkers, R. Guy (Eds.), *Lipophilicity in Drug Disposition: Practical and Computational Approaches to Molecular Properties Related to Drug Permeation, Absorption, Distribution, Metabolism and Excretion*, Univ. Lausanne, 2001, Ch. 22 (in press)] for measuring solubility-pH profiles, using various detection systems.

Classical approaches are based on the so-called saturation shake-flask method, and new rigorous methods are usually validated against it. However, most classical techniques are slow and cannot easily be adapted for the high-throughput needs of modern drug discovery research, one focus of our invention.

At the early stages of research, drug candidate compounds are stored as DMSO solutions, and solubility measurements need to be performed on samples introduced in DMSO, often as 10-30 mM solutions. It is well known that even small quantities of DMSO (<5%) in water can increase the solubility of molecules, and it is a challenge to determine the true aqueous solubility of compounds when DMSO is present, another focus of our invention.

Turbidimetric Ranking Assays

Turbidity detection-based methods, popularized by Lipinski and others [A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, *Adv. Drug Deliv. Rev.* **1997**, *23*, 3-25; P.Q. Charmaine, M.B. Nicholas, A.K. Irwin, *Eur. Pharm. Rev.* **1998**, *3(4)*; C.D. Bevan, R.S. Lloyd, *Anal. Chem.* **2000**, *72*, 1781-1787], in part have met some high-

throughput needs of drug discovery research. The methods, although not thermodynamically rigorous, are an attempt to rank molecules according to expected solubilities. Various implementations of the basic method are practiced at several pharmaceutical companies, using custom-built equipment. Detection systems based on 96-well microtitre plate nephelometers have been introduced recently (e.g., LabSystems: Franklin, MA, USA). The desirable automated solubility analytical device incorporating such a detector requires the operator to develop an appropriate chemistry procedure and to integrate a robotic fluidic system in a customized way. The shortcomings of the turbidity methodology are poor reproducibility (in part due to variability of scattering due to particle size, sometimes erratic scattering due to sedimentation of the sample suspension, and adhesion of suspensions to walls of vessels), the use of excessive amounts of DMSO, and from the view-point of the critical needs of the pharmaceutical industry, the lack of standardization of practice.

HPLC-Based Assays

Several pharmaceutical companies have taken the classical saturation shake-flask method and transferred it onto 96-well plate technology and a robotic liquid dispensing system, in an effort to increase throughput. Analyses are performed with reverse-phase HPLC. Often it is necessary to develop the appropriate chromatographic methods, since in discovery, compounds may not be sufficiently characterized at the early stages. However, generic fast gradient methods may be

eliminating the need for method development. In some companies, the DMSO is first eliminated by a freeze-drying procedure, before the aqueous buffers are added, which adds significantly to the assay time and can be problematic with volatile samples. Chromatographic detection systems, although rich in information, being *serial*, are inherently slow. Data handling is often the rate-limiting step in the operations.

Ideal Solubility - pH Relationships

For ionizable molecules in dilute solutions (where salt precipitation may be ignored), when the log (logarithm) of the measured solubility is plotted vs. pH, the curve is bilinear, with one segment having a zero slope and the other segment having a unit slope (positive for acids and negative for bases). For example, for an acid such as diclofenac, the solubility is at a minimum for pH < 3 (where the acid is largely uncharged), stays nearly constant (zero slope segment) as pH is increased, until the it exceeds the pK_a (3.99), after which the solubility increases by a decade for every increased unit of pH, as more and more of the compound converts from the uncharged state to the more-soluble negatively-charged state (unit slope segment). In a simple aqueous equilibrium system, the pH where the asymptotes of the horizontal and diagonal segments intersect is equal to the pK_a of the solute. Under such ideal circumstances, it is possible to determine the pK_a *and* the intrinsic aqueous solubility, S_o, of the compound from the crossing of the segments [Z.T. Chowhan, *J. Pharm. Sci.* **1978**, *67*, 1257-1260; W.H. Streng, H.G.H. Tan, *Int. J. Pharm.* **1985**, *25*, 135-145].

Such pK_a values can be directly measured by commercially-available devices [C.D. Bevan, A.P. Hill, D.P. Reynolds, Patent Cooperation Treaty, WO 99/13328, 18 March 1999; Sirius Analytical Instruments Ltd., UK].

Complications Which May Thwart the Reliable Measurement of Aqueous Solubility

Certain surface-active compounds when dissolved in water under conditions of saturation form self-associated aggregates or micelles, which can interfere with the determination of the true aqueous solubility and the pK_a of the compound [T.J. Roseman, S.H. Yalkowsky, *J. Pharm. Sci.* **1973**, *62*, 1680-1685; C. Zhu, W.H. Streng, *Int. J. Pharm.* **1996**, *130*, 159-168]. When the compounds are very sparingly soluble in water, excipients are often added to enhance the rate of dissolution. However, the presence of the excipients also can interfere with the determination of the true aqueous solubility. If solubility measurements are done in the presence of simple surfactants [J. Jinno, D.-M. Oh, J.R. Crison, G.L. Amidon, *J. Pharm. Sci.* **2000**, *89*, 268-274], bile salts [S.D. Mithani, V. Bakatselou, C.N. TenHoor, J.B. Dressman, *Pharm. Res.* **1996**, *13*, 163-167], cyclodextrins [P. Li, S.E. Tabibi, S.H. Yalkowsky, *J. Pharm. Sci.* **1998**, *87*, 1535-1537], or ion-pair forming counterions [J.D. Meyer, M.C. Manning, *Pharm. Res.* **1998**, *15*, 188-193], extensive considerations need to be applied in attempting to extract the true aqueous solubility from the data, third focus of our invention.

BRIEF SUMMARY OF THE INVENTION

This invention relates to a method for the determination of solubility of a compound and an analytical device for carrying out said method. The basic method involves determining solubility of a compound by measuring the UV spectrum of a reference solution of the compound, under conditions avoiding or suppressing precipitation, and comparing it to the UV spectrum of a saturated sample solution of the compound. Variations of the basic method include: (a) making reference solutions either by dilution of the sample solution to the point where precipitation is avoided, or making reference solutions by adding a water-miscible cosolvent to the sample solution so that precipitation is suppressed, and comparing the UV absorbances of the compound under reference conditions to the compound in a saturated solution, (b) determining the true aqueous solubility from the effect on the pK_a that results from dissolving the compound in an aqueous solution containing some DMSO (typically 0.1 - 5% v/v), and (c) correcting concentrations determined from the UV absorbance values for impurities and other factors that might affect the shape of the sample absorbance curve taken of the saturated solution.

Solubility determinations are illustrated with 15 sparingly-soluble generic, mostly-ionizable drugs (Fig. 1), covering about three orders of magnitude in solubilities, with the lowest value being about 0.1 µg/mL (terfenadine at high pH). The ionizable bases include amiloride, amitriptyline, chlorpromazine, miconazole, nortriptyline, phenazopyridine, propranolol, and terfenadine. The ionizable acid set consists of

diclofenac, furosemide, indomethacin, 2-naphthoic acid, and probenecid. Piroxicam was picked as an example of an ampholyte, and griseofulvin as an example of a non-ionizable molecule. The results of the solubility measurements were compared to literature equilibrium solubility-pH values from reliable sources, and to values determined by the pSOL Model 3 (*p*ION) acid-base titrator [A. Avdeef, C.M. Berger, C. Brownell, *Pharm. Res.* **2000**, *17*, 85-89].

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

Figure 1 shows the structure of compounds evaluated using the invention and for which data appears herein.

Figure 2 represents a schematic of an analytical device utilized to carry out the methods described in this patent.

Figure 3 is a flow chart showing the aqueous dilution method: (a) general depiction, (b) specific implementation.

Figure 4 is a flow chart showing the cosolvent method: (a) general depiction, (b) specific implementation.

Figure 5 illustrates the solubility - pH curve for a hypothetical ionizable acid with a pK_a of 4 and three different intrinsic solubility values: (a) 5 µg/mL, (b) 50 µg/mL, and (c) 250 µg/mL. In each of the cases, the upper horizontal line represents the maximum possible sample solution concentration, and the lower horizontal line represents the maximum possible reference solution concentration, cf. Fig. 6 below. Intersections of the lines with the curve form the working range of the aqueous dilution method for ionizable compounds.

Figure 6 shows the expected ratios of the areas-under-the-curve, R (equation 5 below), corresponding to the examples in Fig. 5. Solubility values are derived under the conditions $0.05 < R < 1$ (equation 6). Note that 0.05 refers to the 20-fold dilution ratio in the aqueous dilution method.

Figure 7 shows curves generated from data, using equation 6, for solubility as a function of pH for several of the compounds studied by the aqueous dilution method. The horizontal lines represent the maximum possible sample concentration, C_S. The sample solution is not saturated when the discrete points coincide with the horizontal line, indicating that the solubility cannot be determined for those points.

Figure 8 shows for several of the studied molecules the logarithm of the apparent solubility versus pH curves in solutions containing 0.5% (v/v) DMSO (solid line), and the corresponding calculated aqueous solubility versus pH, using equations from Table 1 below (dashed line). The circles represent measured points; the solid line represents the best-flit curve of the measured points, in accordance with the expected shape of a log S vs. pH curve; the dashed line represents the log S vs. pH curve after correction for the anomalies caused by DMSO, aggregation, and the like.

Figure 9 shows UV spectra of piroxicam taken at several values of pH, typical of spectra taken in the aqueous dilution method. Note that the reference (right) absorbances are 20 times lower in optical densities, compared to the highest absorbances of the sample spectra (left).

Figure 10 shows UV spectra of (a) miconazole and (b) phenazopyridine, taken at several values of pH, typical of spectra taken in the cosolvent method. Note that the reference (right) absorbances are slightly *higher* than the highest sample absorbances (left), in contrast to the situation in the aqueous dilution method (Fig. 9). In principle, this indicates that the cosolvent method is more sensitive than the aqueous dilution method.

Figure 11 shows sample and reference spectra, where there is a shape difference in the region of 250-350 nm. The special weighting scheme, equation 27 below, down-weights the data in the region of 285 nm by a factor of 169. Multiplying the reference curve by 10 and superimposing it on the sample curve produced the dashed curve.

Figure 12 shows the alkalimetric pH titration curve of the aqueous universal buffer solution.

Figure 13 shows the alkalimetric pH titration curve of the cosolvent-water universal buffer solution.

DETAILED DESCRIPTION OF THE INVENTION

We have adapted the classical shake-flask method to the *96-well microtitre plate format*, but our detection system is not based on the traditional, one-compound-at-a-time HPLC method. The invention is a *parallel detection method*, based on the use of a 96-well microtitre plate *UV/visible (hence called 'UV') spectrophotometer*. Our invention can measure 96 solubilities at a time, significantly increasing

throughput. Compound-dependent method optimization is not required in the direct UV method.

(1) The essence of our invention is that of making concentration standards under conditions avoiding or suppressing precipitation *for UV* spectrophotometers (in two different implementations: *(a) aqueous dilution* and *(b) cosolvent)*, using molecules, especially ionizable molecules, whose UV spectroscopic properties are unknown at the start of the assay. (Simply weighing the compounds and preparing solutions does not work in general, because the compounds precipitate under conditions necessary to characterize their solubilities.)

(2) Also, a novel and simple computational method is developed to extract the aqueous intrinsic solubilities of drug molecules from data altered by DMSO-drug binding, or drug-drug aggregation reactions, or drug reactions with non-ionizable excipients, using the difference between the apparent pK_a measured by the analytical device and the true pK_a measured by analytical devices available commercially (e.g., from Sirius Analytical Instruments Ltd., Forest Row, E. Sussex, UK).

(3) Also, an improved general method for determining concentration by UV spectrophotometry is derived, based on using a novel peak shape algorithm for adjusting weights in a whole-spectrum weighted regression analysis, matching spectra of reference solutions (of known concentration, under conditions avoiding or suppressing precipitation) to solutions containing analyte of unknown concentration (due to precipitation).

DEFINITIONS OF TERMS

If the molecule is ionizable, its charge depends on the pH of the solution in which it is dissolved. The pH at which the concentration of the *charged* form is equal to the concentration of the *uncharged* form is called the pK_a of the molecule. For example, for the ionizable acid (or ionizable base), represented by HA (or B), chemists usually denote the equilibrium reaction by the equation

$$HA \rightleftarrows A^- + H^+ \qquad (\text{or } BH^+ \rightleftarrows B + H^+) \qquad (1)$$

and the corresponding equilibrium ionization constant is defined as

$$K_a = [A^-][H^+] / [HA] \qquad (\text{or } K_a = [B][H^+]/[BH^+]) \qquad (2)$$

By definition, $pK_a = -\log K_a$. When a solute molecule, HA (or B), is in equilibrium with its precipitated form, HA(s) (or B(s)), chemists usually denote the process by the equilibrium expression

$$HA(s) \rightleftarrows H \qquad (\text{or } B(s) \rightleftarrows B) \qquad (3)$$

and the corresponding equilibrium constant is defined as

$$S_o = [HA]/[HA(s)] = [HA] \quad (\text{or } S_o = [B]/[B(s)] = [B]) \quad (4)$$

By convention, [HA(s)] = [B(s)] = 1. Equations (3) represent the precipitation equilibria of the uncharged species, and are characterized by the *intrinsic*

solubility equilibrium constant, S_o. (The zero subscript denotes the zero charge of the precipitating species.)

The values of the equilibrium constants in equations (2) and (4) depend on the solvent system used (water and aqueous buffers in our case), the inert salt used to adjust the ionic strength of the solution (e.g., NaCl or KCl), temperature (22-25°C in our case), pressure (ambient in our case), and may be affected by organic components (other than the sample) in the aqueous solution, such as DMSO, propanol, surfactants, bile acids, lipophilic counterions, cyclodextrins, and the like. For example, if the solvent system were changed from water to 50% v/v methanol-water, then the pK_as of *acids would be higher* and of *bases would be lower*, compared to values determined in pure water [A. Avdeef, J.E.A. Comer, S.J. Thomson, *Anal. Chem.* **1993**, *65*, 42-49].

We use the term solubility to mean the concentration of a solute (in units of moles per liter, M, or micrograms per milliliter, µg/mL) in a *saturated* pH-buffered aqueous solution. Under pH conditions where the sample molecule is essentially uncharged, the measured solubility is equal to the intrinsic solubility of the molecule. Solubility may depend on pH, but the intrinsic solubility, being a thermodynamic equilibrium constant, does not.

We will implicitly refer to pK_as or log S_o or log S of compounds as values determined in water or an aqueous buffer. When we use the term "apparent" pK_a, or "apparent" log S_o, we do so to emphasize that the aqueous value may not be evident when solutions contain

components such as DMSO, propanol, surfactants, bile acids, lipophilic counterions, cyclodextrins, and the like, that may affect the ionization properties of the compound.

The term "stock" solution will refer to a solution made from a precisely known quantity of pure compound dissolved in a known volume of solvent, usually pure DMSO, usually at a 10-100 mM concentration level. Compounds are expected to be fully dissolved in the stock solution.

We use the term "reference" solution to mean a solution of the sample compound where the concentration of said compound is known. The reference solution can be prepared by precisely weighing a quantity of the pure compound and dissolving it in a precise volume of solvent (usually water or aqueous buffer), or by adding a precise volume of a stock solution of said compound to a precise volume of solvent, *under conditions avoiding or suppressing precipitation*. Two ways are developed to suppress or avoid precipitation: (a) simply make a dilute aqueous solution, below the limit of solubility, or (b) add water-miscible cosolvent to an otherwise saturated aqueous solution.

The term "saturated" solution refers to a solution at equilibrium, containing enough sample compound, so that a portion of the compound is precipitated out of the solution and a portion remains dissolved, at a concentration equal to the solubility. In other words, a saturated solution of a compound contains an excess of undissolved solid form of the compound in contact with its solution.

The term "supersaturated" solution refers to a solution containing no solid and more dissolved compound than expected from its equilibrium solubility. Usually, such solutions are unstable, and given enough time, will precipitate.

By the term "filtration", we broadly mean the separation of the precipitated solid from a saturated solution, either by passing said solution through a filter, by sedimentation, by centrifugation, or by any other separation means.

Saturated solutions are usually "concentrated," and reference solutions are usually "dilute," unless cosolvent is used.

To cause the formation of a saturated solution, a small aliquot of the stock solution (typically 1 - 50 μL) or a weighed quantity of compound is added to an aqueous buffer solution (typically 1000 - 2000 μL), which is called the "system solution" here.

By the term "excipient", we mean any additive in a solution that may affect the solubility of the solute. More specifically, we mean a non-ionizable additive in the pH interval where the sample compound is ionizable. Examples of such additives, as we use the term, are DMSO, surfactants, bile salts, phospholipids, cyclodextrins, ion-pair forming counterions, polymers, cosolvents, and combinations thereof.

COMPONENTS OF THE ANALYTICAL DEVICE OF THE INVENTION

Hardware

The analytical device, shown in a block diagram in Fig. 2, consists of a robotic liquid handling system

(*1*), a microtitre plate scanning UV spectrophotometer (*2*), a pH titrator device (*3*), a microtitre plate vacuum filtration manifold (*4*), a microtitre plate washer (*5*), a microtitre plate orbital shaker (*6*), four precision syringe dispensers (*7*), four dispenser arms positioned by the robot anywhere on the worktable of the liquid handling system (*8*), a wash station and waste trough (*9*), a small-tips pipet rack holder (*10*), a large-tips pipet rack holder (*11*), a used-tip collector (*12*), a stock sample microtitre plate (*13*), a 20-fold diluted stock sample microtitre plate (*14*), a deep-well microtitre plate for saturated solutions (*15*), a deep-well microtitre plate for reference solutions in the aqueous dilution method (*16*), a plastic UV microtitre plate (*17*), four test tubes filled with rinse solution (*18*), a test tube for cosolvent (*19*), a test tube for the DMSO (*20*), a test tube for 0.5 M KOH titrant (*21*), a titration vessel, with a magnetic stir bar and a magnetic stir motor underneath (*22*), a test tube to store the pH electrode (*23*), and an electrode wash station (*24*).

The robotic liquid handling system (*1*), is equipped with four high-precision 0.5 mL syringes equipped with two-way valves (*7*), and four dispenser arms, with conductive-plastic disposable tips (*8*), a liquid-level sensing system, two racks for tips (*10,11*), six microtitre plate holders (*13-17*), dram-size vial and test tube holders (*18-21*), a rinse and waste station (*9*), and approximately 100x50 cm workspace (e.g., Genesis 100/4 System from Tecan, Research Triangle Park, NC, USA).

A 96-well microtitre plate scanning spectrophotometer is a part of the analytical device (*2*), with wavelength range at least 200-850 nm, with <5 nm resolution (e.g., SpectraMAX 190 from Molecular Devices, Sunnyvale, CA, USA).

Although the 96-well microtitre plate format is used preferentially, the method and the analytical device can be constructed with other plate formats, for example, the 384-well microtitre plate format.

Although our preferred detector system is a scanning microtitre plate UV spectrophotometer, diode-array microtitre plate UV spectrophotometers, and flow-through UV detector systems, scanning or diode array, may also be used.

Although a UV detector is the preferred embodiment of the invention, the detector may be any suitable spectrophotometric analytical detector, such as ultraviolet or visible spectrophotometer, a fluorimeter, a colorimeter, a light-scattering device, polarimeter, optical rotation or circular dichroism detector.

A pH titrator (*3*) is used, having a vessel to hold the solution being titrated (*22*), equipped with a pH meter capable of precisely reading pH from 1.5 - 12.5, having a dispenser able to add 0.5 M KOH titrant in precise small amounts, such as 1 µL (*7*), and able to stir the solution during titrant additions (e.g., *p*SOL Model 3 from *p*ION, Woburn, MA, USA).

A vacuum filtration manifold (e.g., from Whatman) for microtitre plates (*4*) is used, with a source of vacuum.

A microtitre plate washer (*5*) is used, with a 75% v/v methanol wash solution (e.g., from Tecan).

A microtitre plate orbital shaker is used (*6*) (e.g., from Lab-Line Instruments, Inc.).

Also used are commercially-sourced 96-well polyethylene/polypropylene microtitre plates, in 0.4 and 2.2 mL well capacities (numerous sources); commercially-source plastic UV plates (e.g., from Corning-Costar or Greiner).

In the basic protocols described below, 8 compounds are sampled in each microtitre plate, at 12 different pH-buffer solutions. This reflects the 8-row x 12-column layout of the microtitre plates. The procedures can be easily scaled up three-fold to 24 samples per day on the present robotic system, still preserving the 12-pH profile, or to 384 samples per day at one pH.

Reagents

A universal buffer system solution is used (*25*), designed to have a linear response to alkali titrant additions in the pH range 3-10, with nonlinearity in pH of about ± 0.05 pH units. The solution possesses buffer components with low-UV absorption (each component with OD < 0.05 at 220 nm for a 4.5 mm path length). The solution possesses buffer components with low tendency to interact with sample species, and specifically excludes phosphate and citrate. A 2-L solution of the buffer, the pH of which is initially near 3, has the capacity of about 100 mL of 0.5 M KOH when pH is raised to 10. The ionic strength of the universal buffer solution is about 0.01 M. Such a universal buffer solution is specifically designed for solubility measurements and is commercially available from *p*ION.

A standardized 0.5 M KOH solution is used (***21***), containing < 0.5% M/M carbonate (available from a number of commercial sources).

Organic solvents are used: spectroscopic grade (or better) DMSO (***20***) and 1-propanol (***19***) (numerous commercial sources).

Software

A computer program is used, which controls the actions of the robotic fluidic delivery system, prompts the operator to perform certain tasks, controls the actions of the spectrophotometer, and processes the spectral data to determine aqueous solubilities as a function of pH and the intrinsic aqueous solubility, displays the data graphically and in report forms, and which transfers the results of the analyses to a Microsoft® Excel spreadsheet. It is commercially available from *p*ION.

The software is loaded on a Windows NT® computer, which connects to the robotic workstation (***1***), the UV spectrophotometer (***2***), and the pH titration device (***3***), by RS232 serial cables, and controls the actions of the analytical device and stores the spectra collected for further processing.

IMPLEMENTATION BASED ON AQUEOUS DILUTION METHOD

The diagram in Fig. 3a shows the general concept of the aqueous dilution method of preparing reference solutions for UV measurements. The "sample" track on the left side of Fig. 3a depicts a series of steps leading up to the taking of the sample spectrum. A known quantity of sample (***27***) is added to a known volume

of the universal buffer solution of known pH (***28***), the amount of said sample being sufficient to cause precipitation to occur in the formed saturated solution (***29***). After waiting a period of time to allow the saturated solution to reach a desired steady state, the solution is filtered (***30***) to remove the solid, to obtain a clear solution (***31***), whose spectrum is then taken by the UV spectrophotometer (***32***). The mathematical treatment of the spectral data (described below) yields the area-under-the-curve of the filtered sample solution (***31***), AUC_S.

The "reference" track on the right side of Fig. 3a depicts a series of steps leading up to the taking of the reference spectrum. A known quantity of sample (***33***) is dissolved in a known volume of the universal buffer solution of known pH (***34***), the amount of said sample being *X-times less* than ***27***, in order to avoid precipitation in the formed solution (***35***). The spectrum is *immediately* taken by the UV spectrophotometer (***36***), to take advantage of the possibility that solution ***35*** may be "*supersaturated*" (that is, solid should have precipitated, but because not enough time was allowed for the solid to precipitate, the solution was temporarily clear and free of solid). The mathematical treatment of the spectral data yields the area-under-the-curve of the reference sample solution (***35***), AUC_R.

When the ratio of the two AUC values,

$$R = AUC_R / AUC_S \qquad (5)$$

is greater than 1, *supersaturation* is indicated, and solubility can be determined by equation (6) below.

When R = 1, the sample and the reference solutions are both saturated, and solubility cannot be determined, *unless* visual inspection of solution **35** indicates that no solid is present, which could happen in a *supersaturated* solution. In this case, solubility can be determined by equation (6) below.

To overcome the situation of R = 1 with both solutions being saturated, a higher value of X needs to be tried, and the procedure repeated until R < 1.

If solubility cannot be determined because R = 1, one can conclude that the *solubility is less than or equal to* the calculated concentration of ***35*** (the calculation ignoring precipitation).

At the other end of the scale of ratios, when R = 1/X, the compound is fully dissolved in both the pre-filtered sample solution (***29***) and the reference solution (***35***), and solubility cannot be determined. (Ideally, R cannot be less than 1/X, unless Beer's law is violated.) However, one can conclude that *solubility is greater than or equal to* the calculated concentration of the solution ***35*** (concentration C_R). To overcome this particular limitation, more sample ***27*** may be used.

When the condition $1/X < R < 1$ is met, then the solubility of the sample compound is

$$S = C_R / R \qquad (6)$$

where C_R is the calculated concentration of ***35***.

In example 1, we implement the aqueous dilution method with the value of X = 20.

IMPLEMENTATION BASED ON COSOLVENT METHOD

The diagram in Fig. 4a shows the general concept of the cosolvent method of preparing reference solutions for UV measurements. The "sample" track on the left side of Fig. 4a depicts a series of steps leading up to the taking of the sample spectrum in a water-cosolvent solution. A known quantity of sample (***37***) is added in a known volume of the universal buffer solution of known pH (***38***), the amount of said sample being sufficient to cause precipitation to occur in the formed saturated solution (***39***). After waiting a period of time to allow the saturated solution to reach a desired steady state, the solution is filtered (***40***) to remove the solid, and obtain a clear solution (***41***).

A volume Y of a water-miscible cosolvent ***42*** is added to a volume Z of solution ***41*** to produce a new solution ***43***, in which the compound is diluted by Z/(Y+Z), from its concentration in solution ***41***. The spectrum of solution ***43*** is then immediately taken by the UV spectrophotometer (***44***), to minimize evaporation of the water-cosolvent solution. The mathematical treatment of the spectral data yields the area-under-the-curve of the filtered cosolvent sample solution (***43***), AUC_S^{COS}.

Suitable cosolvents may be selected from a number of water-miscible organic solvents, such as acetonitrile, methanol, ethanol, iso-propanol, 1-propanol, ethylene glycol, propylene glycol, polyethylene glycol 400 (PEG-400), 1,4-dioxane, dimethylformamide, acetone, tetrahydrofuran, dimethyl-sulfoxide (DMSO), and mixtures thereof. The ones with the lowest vapor pressure, the greatest capability in

dissolving a solute (i.e., highest solubilizing power), and the lowest UV absorption are preferred.

The "reference" track on the right side of Fig. 4a depicts a series of steps leading up to the taking of the reference spectrum in a water-cosolvent solution. A known quantity of sample (***45***) is added to a known volume of the universal buffer solution of known pH (***46***), the amount of said sample being *comparable to that of* ***37***, no effort being made in this step to suppress precipitation in the formed solution (***47***).

A volume Y of a water-miscible cosolvent ***42*** is added to a volume Z of solution ***47*** to produce a new solution ***48***, in which the compound is diluted by Z/(Y+Z), from its concentration in solution ***47***.

It is necessary to ensure that the cosolvent completely dissolves the compound in solution ***48***. If this is not the case, different values of Y and/or Z must be chosen and the whole procedure repeated to ensure that solution ***48*** is solid-free.

The spectrum of solution ***48*** is then immediately taken by the UV spectrophotometer (***49***), to minimize evaporation of the water-cosolvent solution. The mathematical treatment of the spectral data yields the area-under-the-curve of the filtered cosolvent sample solution (***48***), AUC_R^{COS}.

Define the ratio of the two AUC values,

$$R^{COS} = AUC_R^{COS} / AUC_S^{COS} \qquad (7)$$

The solubility of the sample compound is

$$S = (1 + Y/Z)\ C_R^{COS} / R^{COS} \qquad (8)$$

where C_R^{COS} is the calculated concentration of the compound in solution ***48***.

In example 2, we implement the cosolvent method with the value of Z/(Z+Y) = 0.5, using 1-propanol.

METHOD USING pK_a-SHIFTS FOR DETERMINING TRUE AQUEOUS SOLUBILITY OF IONIZABLE COMPOUNDS IN THE PRESENCE OF NON-IONIZABLE CHEMICALS THAT DISTORT THE SOLUBILITY-pH PROFILES

DMSO Binding to the Uncharged Form of a Compound

Although the results depicted in Fig. 7 (plots of experimental S vs. pH) were very satisfactory at first sight, we soon discovered unexpected anomalies, which are not immediately apparent from the figures. The aberrations became evident when log S were plotted vs. pH (Fig. 8).

We found that the log S vs. pH curves (cf., Background section) were altered in the presence of 0.5% v/v DMSO, in that the apparent pK_a values, pK_a^{APP}, derived from log S vs. pH, were different from the true pK_a values by about one log unit. The pK_a^{APP} values were generally *higher* than the true pK_as for ionizable *acids* ("positive" shift), and *lower* than the true pK_as for ionizable *bases* ("negative" shift).

We believe that this is caused by DMSO binding to the drugs. Although we did not anticipate it at first, we actually had collected the data necessary to calculate the extent of drug - DMSO binding, and also to correct for the effect, to obtain true aqueous solubility -pH profiles. For example, equation (9)

represents the binding of the uncharged species by n molecules of DMSO (e.g., in 0.5% v/v).

$$HA + n\ DMSO \rightleftarrows HA(DMSO)_n \quad (9)$$

If the effect of the DMSO is present, and it is ignored in the analysis, then the determined solubility is *not* the value one would measure in the absence of DMSO.

We discovered that if one determines the pK_a^{APP} (in the presence of the DMSO effect) in the solubility assay, and subtracts from it the true pK_a (determined using commercial instruments such as those from Sirius, UK), the difference, when subtracted from the logarithm of the apparent (DMSO-distorted) solubility, S_o^{APP}, yields the true aqueous solubility constant:

$$\log S_o = \log S_o^{APP} - (pK_a^{APP} - pK_a), \quad \text{for ionizable acids} \quad (10)$$

DMSO makes the compound *appear* more soluble, and the true aqueous solubility can be determined from the apparent solubility by subtracting the pK_a difference, as done in equation (10).

Furthermore, we discovered that if one is considering the case of an ionizable base, such as chlorpromazine, the shift of the pK_a is to values *lower* than the true pK_a. The corrective treatment is indicated by equation (11)

$$\log S_o = \log S_o^{APP} + (pK_a^{APP} - pK_a), \quad \text{for ionizable bases} \quad (11)$$

For an amphoteric molecule (which has both acid and base functionality) with two pK_as, either equation (10) or equation (11) may be used, depending on which of the two pK_as is selected.

Fig. 8 illustrates the apparent solubility -pH curve (solid line) and the true aqueous solubility-pH curve (dashed line), correcting for the effect of DMSO for several of the molecules considered.

Uncharged Forms of Compound-Compound Aggregation

Shifts in the pK_a can also be expected if water-soluble aggregates form from the uncharged monomers. This may be expected with surface active molecules or molecules such as piroxicam [J. Jinno, D.-M. Oh, J.R. Crison, G.L. Amidon, *J. Pharm. Sci.* **2000**, *89*, 268-274]. Consider the case where no DMSO is present, but aggregates form, of the sort

$$m\ HA \rightleftarrows (HA)_m \qquad (12)$$

The assumption is that the aggregates are water soluble, that they effectively make the compound appear more soluble, and if ignored, they will lead to the erroneous assessment of solubility. We discovered that equations (10) or (11), for ionizable acids or ionizable bases, respectively, applies also to the case of aggregation.

Compound-Compound Aggregation of Charged Ionizable Bases

Consider the case of an ionizable base, where the protonated, positively charged form self-associates to form aggregates, but the uncharged form does not. We may have this case with phenazopyridine (Fig. 8). Phenazopyridine is a base which consistently shows *positive* shifts in its apparent pK_a, the opposite of what's expected of *uncharged*-compound DMSO/aggregation effects. A rationalization of this effect can be based on the formation of partially protonated aggregates (perhaps micelles). For example, one can hypothesize that one of the species is $(BH^+)_n$.

$$n\ BH^+ \rightleftarrows (BH^+)_n \qquad (13)$$

We discovered that for such a case, the observed solubility-pH curve is shifted horizontally (not vertically, as with uncharged-compound DMSO/aggregation effects), and that the apparent *intrinsic* solubility is *not* affected by the phenomenon.

Compound-Compound Aggregation of Charge Ionizable Acids

Consider the case of an ionizable acid, where the de-protonated, negatively-charged form, is strongly self-associated, but the uncharged form is not. We may have this case with prostaglandin F2α [T.J. Roseman, S.H. Yalkowsky, *J. Pharm. Sci.* **1973**, *62*, 1680-1685]. Prostaglandin is an ionizable acid with a reported negative shift in its apparent pK_a, the opposite of what's expected of *uncharged*-compound DMSO/aggregation effects. A rationalization of this effect can be based on the formation of partially deprotonated aggregated complexes (perhaps micelles). One can hypothesize a reaction similar to equation (12), but involving negatively-charged ions. The reported solubility-pH curve is shifted horizontally (not vertically, as with uncharged-compound DMSO/aggregation effects), and that the apparent *intrinsic* solubility is not affected by the phenomenon.

Ionizable Compound Binding by Non-ionizable Excipients

We have concluded that a number of phenomena, similar to those of reactions (9), (12), and (13), will shift the apparent pK_a in a manner of the above discussions. For example, the additives in drug formulations, such as surfactants, bile salts, phospholipids, ion-pair forming counterions, cyclodextrins or polymers may make the drug molecule appear more soluble. As long as such excipients do not undergo a change of charge state in the pH interval of interest (i.e., the excipients are effectively non-ionizable), and the drug molecule is ionizable in the pH interval of interest, the difference between the apparent pK_a and the true pK_a will reveal the true aqueous solubility, as if the excipient were not present.

Table 1 summarizes some of the relationships developed between solubility, pK_a, and pK_a^{APP}.

TABLE 1 True Aqueous Solubility Determined from pK_a Shifts of Monoprotic Compounds

Ionizable Compound Type	$\Delta = pK_a^{APP} - pK_a$	true aqueous log S_o	Examples (from Fig. 8 and literature)
acid	$\Delta > 0$	log $S_o^{APP} - \Delta$	diclofenac, furosemide, indomethacin, probenecid, naphthoic acid
acid	$\Delta <$ or $= 0$	log S_o^{APP}	prostaglandin F2α [a]
base	$\Delta >$ or $= 0$	log S_o^{APP}	phenazopyridine
base	$\Delta < 0$	log $S_o^{APP} + \Delta$	amitriptyline, chlorpromazine, miconazole, terfenadine

[a] T.J. Roseman, S.H. Yalkowsky, *J. Pharm. Sci.* **1973**, *62*, 1680-1685.

AREA-UNDER-THE-CURVES (AUC) DETERMINED BY WEIGHTED REGRESSION METHOD

The method utilized by the analytical device for determining concentrations of species by UV spectroscopy is based on a weighted regression analysis of whole spectra. All the 65-130 measured absorbances from each microtitre plate well may be used in the analysis. The concentration of the reference species is known, and the object of the analysis is to assess the unknown concentration of the sample in the filtered solution, by appying Beer's law.

The calculated absorbance of the sample species at a particular wavelength i, a_i^{calc}, can be related to the absorbance values of the reference species, a_i^{REF}, the absorbance of DMSO (scaled to the units of the DMSO concentration in the reference well), $a_i^{DMSO(REF)}$, and the absorbance of the sample solution "blank" (buffers and the plastic microtitre plate), $a_i^{BLK(SAMPLE)}$:

$$a_i^{calc} = p_0\, a_i^{REF} + p_1\, a_i^{DMSO(REF)} + p_2\, a_i^{BLK(SAMPLE)} \qquad (14)$$

where p_0, p_1, and p_2 are unknown parameters. In turn, the absorbance of the reference species is derived from the observed reference spectrum, corrected for DMSO and blank:

$$a_i^{REF} = a_i^{REF(TOTAL)} - a_i^{DMSO(REF)} - a_i^{BLK(REF)} \qquad (15)$$

The 'reference' spectrum is that of the compound of known concentration, and the 'sample' spectrum is that of the same compound, but of unknown concentration. At each desired wavelength i, the value of $a_i^{DMSO(REF)}$ is independently established: the DMSO absorbance curve is characterized from measurements of solutions containing all components except for the sample species. The 'blank' absorbances are measured before each assay, by sampling the microtitre plate filled only with the universal buffer solution. So, $a_i^{BLK(REF)}$ and $a_i^{BLK(SAMPLE)}$ are then known.

The object of the analysis is to determine the three unknown parameters p_0, p_1, and p_2. (The parameter p_0 is used directly in solubility determination; usually the other two parameters are determined to have near

unit values.) This is done by weighted linear regression analysis [P. R. Bevington, "Data Reduction and Error Analysis for the Physical Sciences," McGraw-Hill: New York, 1969, pp. 164-186], as outlined below.

Define a column-vector of the unknown parameters, $\mathbf{p} = (p_0, p_1, p_2)$. Define a row-vector, $\mathbf{b}_i$, at each wavelength i, consisting of the three independent parameters a_i^{REF}, $a_i^{DMSO(REF)}$, and $a_i^{BLK(SAMPLE)}$,

$$\mathbf{b}_i = (a_i^{REF}, a_i^{DMSO(REF)}, a_i^{BLK(SAMPLE)}) \qquad (16)$$

so that equation (14) may be expressed in vector notation as

$$a_i^{calc} = \mathbf{b}_i \mathbf{p} \qquad (17)$$

For all the measured wavelengths i (typically, i=0 to N-1, where N=number of wavelengths measured), equation (17) may be expressed in an matrix notation,

$$\mathbf{a}^{calc} = \mathbf{B} \mathbf{p} \qquad (18)$$

Without the subscript, $\mathbf{a}^{calc}$ denotes the column-vector of N rows (the number of discrete wavelengths measured), and $\mathbf{B}$ denotes the N-rows x 3-columns matrix composed of the N individual $\mathbf{b}_i$ vectors.

The parameters $\mathbf{p}$ are determined by finding a solution which minimizes the sum of weighted residuals

$$\begin{aligned} Q &= \sum_{i=0..N-1} (a_i^{obs} - a_i^{calc})^2 / \sigma_i^2 \\ &= \mathbf{a}^{obs,T} \mathbf{W} \mathbf{a}^{obs} - \mathbf{p}^T \mathbf{B}^T \mathbf{W} \mathbf{a}^{obs} - \mathbf{a}^{obs,T} \mathbf{W} \mathbf{B} \mathbf{p} \\ &\quad + \mathbf{p}^T \mathbf{B}^T \mathbf{W} \mathbf{B} \mathbf{p} \end{aligned} \qquad (19)$$

where a_i^{obs} is the measured absorbance of the sample at wavelength i (dependent variable), σ_i^2 is the estimated variance in the measured dependent variables a_i^{obs}, T is the transpose operator, and **W** is the diagonal matrix of weights, each i^{th} element being the inverse variance, $1/\sigma_i^2$. The model equation, a_i^{calc}, is a function of the three **p** parameters, as well as the independent variables, λ_i (i^{th} wavelength) and $\mathbf{b}_i$.

The elements of **p** are determined by setting the derivative of Q with respect to $\mathbf{p}^T$ to zero and solving for **p**.

$$\mathbf{p} = (\mathbf{B}^T \mathbf{W} \mathbf{B})^{-1} (\mathbf{B}^T \mathbf{W} \mathbf{a}^{obs}) \quad (20)$$

Note that p_0 may be substituted into equations (5) and (7) as,

$$p_0 = AUC_S / AUC_R \quad (21)$$

WEIGHTING SCHEME BASED ON PEAK SHAPE ANOMALIES

The method utilized by the analytical device for determining concentrations of species by UV spectroscopy is based on a weighted regression analysis of whole spectra, as described in the last section. The technique is unique in how it assigns weights to the data, to make the assessment of concentrations more reliable, especially when samples are not entirely pure.

The normal weighting scheme used in equation (19) is constructed from the variances

$$\sigma^2(a) = \sigma_c^2 + (\sigma_\lambda \, da/d\lambda)^2 \qquad (22)$$

In the software of the analytical device, σ_c = 0.0002 (absorbance units), the fixed contribution to the variance in the measured absorbance (experimentally determined by replicate baseline measurements, usually in the domain 450-500 nm), and σ_λ = 0.2 nm (estimated error in wavelength, specified by the UV spectrophotometer manufacturer). The dependent variables, a_i^{obs}, and the independent variables, λ_i, are all assumed to be subject to error. The weighting scheme properly recognizes that measurements of absorbance on the steep sides of peaks are not as reliable as those near the peak top or from the baseline portions.

However, suppose that the sample spectrum has an "impurity" absorption (or scattering due to dust particles) at a particular wavelength, which is not found in the reference spectrum. If the impurity portion in the sample spectrum were not treated in any special way, then the procedure of the previous section would overestimate p_0, due to the added contribution of the impurity absorbance.

Consider a sample solution containing a mixture of components (i.e., impurities). The observed spectrum would be a manifold of several contributing spectra. It is reasonable to assume that the solubilities of all the components are different. If a saturated solution is made of the mixture, some components will precipitate and some will not. The consequence of this is that the UV spectrum of the manifold may change in shape as a result of the precipitation. The shape-based weighting

scheme can detect such anomalies, minimizing their effect on the p-parameter determination.

The calculation scheme assesses the peak maximum, a_{max}^{REF}, and the wavelength, λ_{max}, at which the maximum occurs for the reference spectra. For any given wavelength i, the following reference and sample ratios are calculated:

$$r_i^{REF} = a_i^{REF} / a_{max}^{REF} \quad (23)$$

$$r_i^{SAMPLE} = a_i^{SAMPLE} / a_{max}^{SAMPLE} \quad (24)$$

where a_{max}^{SAMPLE} is the sample absorbance at λ_{max}. If the peak in the sample had the identical shape as the peak in the reference solution, then the two ratios, (23) and (24) would be the same. However, if it happens that the impurity absorbance is at its maximum at the particular wavelength i, then the reference ratio would be greater than the sample ratio.

Define a constant that is the ratio of the two above ratios:

$$s_i = | r_i^{SAMPLE} / r_i^{REF} | \quad (25)$$

Furthermore, define the empirically-optimized scale factor k_i:

$$k_i = 10^{+6.3\ (s\ -\ 1)} \quad \text{for } s_i > 1 \quad (26a)$$

or

$$= 10^{+6.3\ (1/s\ -\ 1)} \quad \text{for } s_i < 1 \quad 26b)$$

now with this model, the normal weighting scheme in equation (19) is modified:

$$\sigma_{shape}^2(a) = k^2 [\sigma_c^2 + (\sigma\lambda \, da/d\lambda)^2] \quad (27)$$

Higher errors are ascribed to the spectral regions exhibiting impurities. Hence data from the regions of the impurity are made less important in the derivation of the parameter vector **p**, equation (20).

The way that the scale factor, k, is defined, it doesn't matter whether the impurity is in the sample or the reference spectrum: any differences in shape between the two spectra automatically down-weight the region of the differences in the regression equation (20), placing more emphasis on the spectral regions with the common shape.

The following examples are intended to illustrate, but not limit, the invention disclosed herein.

Example 1. Apparatus of Figs. 2 and 3: Solubility Determined by the Aqueous Dilution Method

Fig. 2 summarizes the apparatus detailed below. The general aqueous dilution method used to determine solubility is described in Fig. 3a, and a specific embodiment is highlighted in Fig. 3b and detailed below.

Data Collection

1. The analytical device places 2.5 mL of universal buffer solution (***25***), initially at pH 3, into the side arm tube of a tall test tube ***22*** ("J-tube")

located in the pH titrator assembly (***3***). An alkalimetric pH titration is performed.

2. Analysis of the titration data produces the pH values corresponding the titrant volumes 8, 16, 23, 30, 37, 44, 52, 59, 65, 73, 81 µL; these correspond closely to pH settings 3.0 - 8.5, in increments of 0.5 pH units. These volumes will be used by the device below.

3. The 96-well stock plate is placed in position ***13*** (**S** in Fig. 3b), furthest from operator on left rack on the robot table. Only one column of 8 sample wells will be used in this particular assay; for example, wells A1, B1, ..., H1 (or A2, B2, ..., H2, etc.) will contain the required compounds, 100 mM in DMSO in this example. This is the sample stock plate.

4. A new microtitre plate (must be inert to DMSO -- NUNC 0.5mL polypropylene is satisfactory) is placed in position ***14*** (**Sd** in Fig. 3b) rear position of the rack on the right. This is the empty reference stock plate.

5. A new 96-well deep plate (2.2 mL wells) is placed at position ***15*** (**Dc** in Fig. 3b), middle of the rack on the left on robot table.

6. A new 96-well deep plate (2.2 mL wells) is placed at position ***16*** (**Dd** in Fig.3b), middle of the rack on the right on robot table.

7. A new 96-well Costar UV plate (or Greiner, but Costar is slightly better) is placed in position ***17*** (**U**), nearest to operator on left the rack on robot table.

8. A new rack of 20-µL disposable (conductive) pipet tips is placed into position ***10*** and a new rack of 200-µL disposable (conductive) pipet tips is placed in position ***11*** on the robot table.

9. A plastic waste bag is attached to the bottom of the waste-tip slide (***12***).

10. The system solution bottle needs to have at least 500 mL of the universal buffer solution.

11. A clean test tube in position ***20***, third test tube position nearest the operator of the test tube rack, is filled with 21 mL of DMSO. A clean test tube in position ***21***, second test tube position nearest the operator, is filled with 10 mL freshly prepared, low-carbonate, 0.5 M KOH.

12. Four clean test tubes, each containing 10 mL distilled water, are placed into positions ***18***.

13. The robot loads 1000 µL universal buffer solution into each of the deep 96-well plate in position ***13*** (**Dc** in Fig. 3b) on the table. Then the robot draws 0.5 M KOH from test tube in position ***21*** of the test tube rack. The robot proceeds to deposit 8 µL into the 8 deep wells A2-H2, 16 µL into deep wells A3-H3, 23 µL into deep wells A4-H4, 30 µL into deep wells A5-H5, 37 µL into deep wells A6-H6, 44 µL into deep wells A7-H7, 52 µL into deep wells A8-H8, 59 µL into deep wells A9-H9, 65 µL into deep wells A10-H10, 73 µL into deep wells A11-H11, and 81 µL into deep wells A12-H12.

14. Robot loads an additional 1000 µL universal buffer solution (***25***) into each of the deep wells, followed by *extensive* mixing.

15. Before any sample is added to ***15*** (**Dc**), the robot transfers 150 µL buffer solutions from the deep well to the UV plate in position ***17*** (**U**) on the table (**Dc --> U**). The operator is prompted to take UV spectra of the buffer-filled UV plate, using the spectrophotometer

(*2*). Afterwards, the UV plate is rinsed with methanol-water using the plate washer (*5*) and returned to position *17* (**U**), covered with a lid to keep dust out. This corresponds to the "blank" reading in equations (14) and (15).

16. To each of the deep wells (containing 1850-1931 µL pH buffer), 5 µL sample (**27**) is added, using the 20 µL tips ***13-->15*** (**S --> Dc**). The first sample pickup is discarded back into the stock plate, to ensure that the tip does not have an air gap at the opening and to ensure the dispenser stepper motor gears are free of slack, and that the inside surface of the tip is wetted before subsequent sample pickup. Vigorous "regurgitative" mixing follows. A protective cover is placed over *13* (**S**) plate after the transfers are complete.

17. The top of the deep well plate *15* (**Dc**) is sealed (aluminum plate seals), and the plate is placed on the orbital shaker (*6*) for 4 hours.

18. The robot transfers 190 µL DMSO to the empty reference stock plate ***20-->14***. The robot transfers 10 µL samples: ***13-->14*** (**S --> Sd**). Solutions in **Sd** are thoroughly mixed. The sample stock plate *13* (**S**) may now be removed, protectively sealed, and stored in the refrigerator.

19. The robot loads 1000 µL universal buffer solution (*25*) into each well of the deep 96-well plate in position *16* (**Dd**) on the table. Then the robot draws 0.5 M KOH from test tube in position *21* of the test tube rack and proceeds to deposit 8 µL into the 8 deep wells A2-H2, 16 µL into deep wells A3-H3, 23 µL into deep

wells A4-H4, 30 µL into deep wells A5-H5, 37 µL into deep wells A6-H6, 44 µL into deep wells A7-H7, 52 µL into deep wells A8-H8, 59 µL into deep wells A9-H9, 65 µL into deep wells A10-H10, 73 µL into deep wells A11-H11, and 81 µL into deep wells A12-H12.

20. The robot loads an additional 1000 µL universal buffer solution (***25***) into each of the deep wells, followed by *extensive* mixing.

21. The robot discards 150 µL of each of the deep well solutions to waste. A second blank is not needed, but volumes in the deep well plate ***16*** (**Dd**) need to be the same those of ***15*** (**Dc**) after step 13.

22. To each of the deep wells, 15 µL DMSO are added ***20-->16***.

23. To each of the deep wells (containing 1850-1931 µL pH buffer), 5 µL 20-fold diluted sample are added ***14-->16*** (**Sd --> Dd**). Vigorous mixing follows, a critical step.

24. As soon as mixing is complete in step 23, the robot transfers 150 µL of the reference solution to the UV plate ***16-->17*** (**Dd -->U**). Filtration is not necessary. The operator is prompted to take UV spectra of the UV plate, using ***2***. The deep well plate in position ***16*** (**Dd**) is no longer used. The UV plate is thoroughly washed on the plate washer ***5*** (using 75% methanol - 25% water) and placed in position ***17*** (**U**), with a protective dust cover.

25. After the 4 hour shaking period elapses, the operator transfers the deep well plate from the orbital shaker to position ***15*** (**Dc**).

26. The operatcr places a used 96-well deep plate (2.2 mL wells) in the bottom of the vacuum manifold, position ***4*** on the robot table. On top of that the operator places a new 96-well deep *filter plate* (2.2 mL wells). The operator assembles the vacuum manifold accordingly. The robot transfers 20 µL precipitated solutions for the filtration step: ***15-->4***. The operators is prompted to turn on the vacuum, to filter the solutions. This step pre-conditions the filters with sample, and is part of the so-called "double filtration" step.

27. The operator replaces the bottom (used) deep-well plate with a clean empty 96-well deep plate (2.2 mL wells) in the bottom of the vacuum manifold ***4***. On top of that the operator returns the sample-preconditioned 96-well deep filter plate (2.2 mL wells). The operator assembles the vacuum manifold again.

28. The robot transfers 250 µL saturated sample solutions for the filtration step: ***15-->4***. Afterwards, the deep well filter plate in position ***15*** (**Dc**) may be sealed and stored for future examination or re-use.

29. The operators is prompted to turn on the vacuum, to filter the solutions, some of which may have solids in them. The operator discards the top deep-well filter plate and exposes the bottom deep-well plate for robotic access.

30. The operator removes cover on the UV plate at position ***17*** (**U**). The robot transfers 150 µL to the UV plate ***4-->17***. The operator is prompted to take UV spectra of the UV plate. The deep well plate in position ***4*** may be discarded afterwards. If the plate ***15*** (***Dc***) is to be re-sampled later, then the UV plate should

be thoroughly washed on the plate washer ***5*** (using 75% methanol - 25% water) and placed in position ***17*** (**U**), with a protective cover, for future use. Blank and reference spectra need not be repeated in such re-samplings.

Data Processing

Fig. 9 shows the measured absorption spectra due to piroxicam. The shown absorption spectra had been corrected for the absorption of "blank" plate, and for the absorption due to DMSO (equation 14).

For ionizable compounds, spectra can change dramatically as a function of pH. Also, at a given pH, if a portion of the sample precipitates, the optical density (OD) will decrease, following Beer's law.

Fig. 9 shows how spectra change with pH in the sample and reference solutions of piroxicam, a molecule which is effectively an ionizable acid if only the pH > 3 is considered, with a pK_a of 5.07 (Table 2). Both the sample spectra and the reference spectra are of excellent quality; however, one notes that the optical densities of the reference absorbance curves are relatively low (about OD 0.06 at the maximum), compared to those of the sample. If we had started out with 10 mM DMSO stock solutions, instead of 100 mM, the ODs would not have exceeded 0.006, a useable value, but one nearing the limit of detection. To produce a reference solution free of precipitate, it is necessary to compromise on sensitivity. Example 1 conditions have been demonstrated to work well. However, if 10 mM DMSO stock solutions are used, different DMSO stock plate dilutions need to be considered (**S --> Sd**), along with different sampling volumes (**S --> Dc, Sd --> Dd**).

Example 2 below will show how the sensitivity issue can be avoided by using cosolvents.

As precipitation takes place to varying degrees in different pH values, the spectra of the sample solutions change in optical densities. This can be clearly seen

in Fig. 9, where the sample spectra have the lowest OD values at pH 3.5 and systematically show higher OD values as pH is raised. The changing OD values indicate that solubility changes with pH.

The reference solutions only changes as a result of the ionization of the compound, and not due to changes in concentration of the compound. Usually ionization does not produce large changes in the OD values as a function of pH.

In the above example, the calculated reference solution concentrations in plate ***16*** (**Dd** in Fig. 3b or **35** in Fig. 3a) are

$$C_R = 10/\ (190+10)\ x\ 5\ /\ (2000+V_{KOH}\ -150+5+15)\ x\ 100\ mM$$
$$= 12.8 - 13.4\ \mu M \qquad (28)$$

where V_{KOH}, the volume of 0.5 M KOH added to adjust the pH, varies from 0 to 81 µL.

The reference concentrations are indicated by the lower horizontal lines in Fig. 5.

The *limiting* sample concentrations (assuming no precipitation had taken place in the deep-well plate ***15*** (***31*** in Fig. 3a or **Dc** in Fig. 3b) are calculated according to the formula

$$C_S = 5\ /\ (2000 + V_{KOH} - 150 + 5)\ x\ 100\ mM$$
$$= 258 - 270\ \mu M\ \text{(assuming no precipitate forms)} \qquad (29)$$

The limiting concentration is represented by the horizontal lines in Fig. 7 and the upper horizontal lines in Fig. 5.

The next step requires that the spectra taken from 190 to 500 nm (in increments of 2 nm) be analyzed, to determine the area-under-the-curve (AUC) values, using equation (20) incorporating the shape-based weighting scheme, equation (27), in the analysis. With plastic UV plates, usually only the data in the interval 240 - 500 nm are actually used in the analysis. The optimized regression parameter is related R, the ratio of AUC values, equation (5).

If R = 1 and the reference solution is not supersaturated, then one cannot report the solubility of the compound at the particular pH, since both the sample and the reference solutions have precipitation in them. This is indicated hypothetically in Fig. 5A for pH < pH_R, and in Fig. 6A by the low-pH region flat portion of the curve.

This situation is seldom seen in practice, since the quick handling of the reference plate and the higher amount of DMSO used in the reference plate compared to the sample plate almost always ensures that the sparingly soluble compounds are supersaturated. This is made more probable by the use of 1.06% v/v DMSO in the reference plate, compared to the usual 0.26% v/v DMSO in the sample deep well plate (in the latest variant of the protocol). On the other hand, the sample plate is allowed to sit for at least 4 hours, ample time in most cases for precipitation to start. The longer the wait time, the more "crystalline" the precipitated solid becomes.

An earlier version of the protocol used 0.5% v/v DMSO in both the reference and the sample deep well plates. The latest protocol (1.06% v/v DMSO in

reference and 0.26% in sample) enhances the chances of supersaturation conditions in the reference deep well plates.

If R = 10 / (190+10) = 0.05, then one cannot report the solubility of the compound at the particular pH, since both the sample and the reference solutions have no precipitate. This condition is common, and is indicated for all pH in Fig. 7A, for pH < 8 in Fig. 7B, for pH < 7 in Fig. 7C, for pH > 6 in Fig. 7D, and for pH > 6 in Fig. 7D. This corresponds to the hypothetical conditions for pH > pH_S in Fig. 5A and Fig. 5B, and flat regions at the lowest parts of the curves in Fig. 6. To remedy this, one needs to use more concentrated DMSO stock solutions. This is not a problem in practice, since many discovery applications are mainly concerned with C_S < 50 μg/mL. We demonstrated the aqueous dilution method with C_S of 100 and 200 μg/mL (Fig. 7).

The R vs. pH relationships (Fig. 6) can be automatically assessed by the analytical device, and when the R is less than 1 but greater than 0.05, solubility is calculated according to equation (6). The useful pH domain for solubility determination are indicated in Fig. 5 as pH_R < pH < pH_S. In Fig. 7B, solubilities of amitriptyline can be determined for pH > 8; in Fig. 7C, solubilities can be determined for pH > 7, etc.

The minimum possible solubility refers to the intrinsic solubility, and such S_o values often need to be extrapolated from the S vs. pH curve, taking its theoretical expected shape into account. The apparent intrinsic solubilities, S_o^{APP}, determined in this way are listed in Table 2, column 3, for the compounds used in

the example. All the S_o^{APP} values reported in Table 2 were determined in the presence of 0.5% v/v DMSO, except for phenazopyridine, where 0.26% was used.

Results of Aqueous Dilution Application

The most insoluble compounds were found to be terfenadine, miconazole, indomethacin, chlorpromazine, and piroxicam. We saw no evidence of precipitation with amiloride and nortriptyline, which suggests their solubilities are greater than 208 μg/mL and 98 μg/mL, respectively, in 0.5% v/v DMSO. Although we did see a few crystals in the propranolol well at pH 9.5, we were not able to derive the solubility with any confidence. We estimate that the solubility of propranolol is about 100 μg/mL in the presence of 0.5% v/v DMSO.

Example 2. Apparatus of Figs. 2, 4a, and 4b: Solubility Determined by the Cosolvent Method

Fig. 2 summarizes the apparatus detailed below. The general cosolvent method used to determine solubility is described in Fig. 4a, and a specific embodiment is highlighted in Fig. 4b and detailed below. In the variation utilizing cosolvent, all spectra were collected of solutions containing 50% v/v 1-propanol, but in other respects the methods are very similar.

Data Collection

1. The analytical device places 2.5 mL of universal buffer solution (***25***), initially at pH 3, into the side arm tube of a tall test tube ***22*** ("J-tube") located in the pH titrator assembly (***3***). An alkalimetric pH titration is performed.

2. Analysis of the titration data produces the pH values corresponding the titrant volumes 8, 16, 23, 30, 37, 44, 52, 59, 65, 73, 81 µL; these will correspond closely to pH settings 3.0 - 8.5, in increments of 0.5 pH units. These volumes will be used by the device below.

3. The 96-well stock plate is placed in position ***13*** (**S** in Fig. 4b), furthest from operator on left rack on the robot table. Only one column of 8 sample wells will be used in this particular assay; for example, wells A1, B1, ..., H1 (or A2, B2, ..., H2, etc.) will contain the required compounds, 100 mM in DMSO in this example. This is the sample stock plate.

4. A new microtitre plate (must be inert to DMSO -- NUNC 0.5mL polypropylene is satisfactory) is placed in position ***14*** (**Sd** in Fig. 4b) rear position of the rack on the right. This is the empty reference stock plate.

5. A new 96-well deep plate (2.2 mL wells) is placed at position ***15*** (**Dc** in Fig. 4b), middle of the rack on the left on robot table.

6. Test tube in position ***19*** (***42*** in Fig. 4a) is filled with 20-21 mL spectroscopic-grade 1-propanol.

7. A new 96-well Greiner UV plate (Greiner is better than Costar in the cosolvent method) is placed in position ***17*** (**U**), nearest to operator on left the rack on robot table.

8. A new rack of 20 µL disposable (conductive) pipet tips is placed into position ***10*** and a new rack of 200 µL disposable (conductive) pipet tips is placed in position ***11*** on the robot table.

9. A plastic waste bag is attached to the bottom of the waste-tip slide (***12***).

10. The system solution bottle needs to have at least 500 mL of the universal buffer solution.

11. A clean test tube in position ***20***, third test tube position nearest the operator of the test tube rack, is filled with 21 mL of DMSO. A clean test tube in position ***21***, second test tube position nearest the operator, is filled with 10 mL freshly prepared, low-carbonate, 0.5 M KOH.

12. Four clean test tubes, each containing 10 mL distilled water, are placed into positions ***18***.

13. The robot loads 1000 µL universal buffer solution into each of the deep 96-well plate in position ***13*** (**Dc**) on the table. Then the robot draws 0.5 M KOH from test tube in position ***21*** of the test tube rack. The robot proceeds to deposit 8 µL into the 8 deep wells A2-H2, 16 µL into deep wells A3-H3, 23 µL into deep wells A4-H4, 30 µL into deep wells A5-H5, 37 µL into deep wells A6-H6, 44 µL into deep wells A7-H7, 52 µL into deep wells A8-H8, 59 µL into deep wells A9-H9, 65 µL into deep wells A10-H10, 73 µL into deep wells A11-H11, and 81 µL into deep wells A12-H12.

14. Robot loads an additional 1000 µL universal buffer solution (***25***) into each of the deep wells, followed by *extensive* mixing.

15. Before any sample is added to ***15*** (**Dc**), the robot transfers 73 µL buffer solutions from the deep well to the UV plate in position ***17*** (**U**) on the table (**Dc --> U**); the robot transfers 72 µL of 1-propanol from ***19***. The robot mixes the solvents in each well.

16. The operator is prompted to take UV spectra of the cosolvent-buffer-filled UV plate, using the

spectrophotometer (***2***). The solution is SAVED and the plate is returned to position ***17*** (**U**), with its top *tightly* covered, to keep the dust out and *evaporation* minimized.

17. The robot transfers 190 µL DMSO to the empty reference stock plate ***20-->14***. (1-propanol is not suitable at this point because of its volatility.) The robot transfers 10 µL samples using the 20 µL tips: ***13-->14*** (**S --> Sd**). Solutions in ***14*** (**Sd**) are thoroughly mixed.

18. As soon as the solutions are mixed in step 17, the robot transfers 5 µL from the diluted sample stock solution (using the 20 µL tips) to the blank solutions whose spectra had just been read in step 16: ***14-->17*** (**Sd -> U**). There are now 150 µL in each well of the Greiner plastic UV plate. The diluted sample stock plate ***14*** (**Sd**) may now be removed, protectively sealed, and stored in the refrigerator.

19. The operator reads the UV of the plate with the spectrophotometer ***2***. This becomes the reference plate reading. Discard the solutions and wash plastic UV plate thoroughly on the plate washer ***5*** (75% methanol - 25% water). Place empty clean UV plastic plate in position ***17*** (**U**). Temporarily cover with lid to keep dust out.

20. To each of the deep wells at ***15*** (**Dc**) (containing 1927-2008 µL pH-adjusted universal buffer), 5 µL sample is added, using the 20 µL tips ***13-->15*** (**S --> Dc**). The first sample pickup is discarded back into the stock plate, to ensure that the tip does not have an air gap at the opening and to ensure the

dispenser stepper motor gears are free of slack, and that the inner walls of the tip are wetted with sample. Vigorous "regurgitative" mixing follows. The sample stock plate ***13*** (**S**) may now be removed, protectively sealed, and stored in the refrigerator.

21. Seal the top of the deep well plate (aluminum plate seals), and place on the orbital shaker (***6***) for 4 hours.

22. The operator places a *used* 96-well deep plate (2.2 mL wells) in the bottom of the vacuum manifold (***4***). On top of that the operator places a *clean* 96-well deep *filter plate* (2.2 mL wells). The operator assembles the vacuum manifold accordingly. The robot transfers 20 µL precipitated solutions for the filtration step: ***15-->4***. The operators is prompted to turn on the vacuum, to filter the solutions. This step, a part of the "double filtration" operation, pre-conditions the filters with sample.

23. The operator replaces the bottom (used) deep-well plate with a *new* 96-well deep plate (2.2 mL wells) in the bottom of the vacuum manifold. On top of that the operator returns the sample-preconditioned 96-well deep filter plate (2.2 mL wells). Operator assembles the vacuum manifold again.

24. The robot transfers 250 µL precipitated sample solutions for the filtration step: ***15-->4***. Afterwards, the deep well filter plate in position ***15*** (**Dc**) may be stored for future examination or re-use.

25. The operators is prompted to turn on the vacuum, to filter the solutions, some of which may have solids in them. The operator discards the top deep-well

filter plate and exposes the bottom deep-well plate for robotic access.

26. The operator removes cover on the UV plate at position ***17*** (**U**). The robot transfers 75 µL of the filtered sample solution to the UV plate: ***4-->17***. Immediately following that, the robot transfers 75 µL 1-propanol to the UV plate: ***19-->17***. The solutions are thoroughly mixed.

27. The operator is prompted to take UV spectra of the UV plate (***17-->2***). The UV plate and the deep well plate in position **4** are discarded afterwards.

Data Processing

Fig. 10a shows the measured absorption spectra of miconazole, and Fig. 10b shows those of phenazopyridine. The shown absorption spectra had been corrected for the absorption of the "blank" plate, and for the absorption due to DMSO (equation 14).

For ionizable compounds, spectra can change dramatically in shape as a function of pH, as in the reference spectra in Fig.10b, but this isn't always so, as illustrated by the spectra of miconazole in Fig.10a.

The reference solutions only changes as a result of the ionization of the compound, and not due to changes in concentration of the compound.

Also, at a given pH, if a portion of the sample precipitates, the optical density (OD) will decrease, following Beer's law, which is what all the sample spectra in Figs. 9 and 10 show.

Fig. 10a shows how spectra change with pH in the sample solutions of miconazole, an ionizable base with a pK_a 6.07 (Table 2). Both the sample spectra and the reference spectra are of excellent quality, with the optical densities of the reference absorbance curves being comparable to those of the sample. This is one of the advantages of the cosolvent method over the aqueous dilution method, in that sensitivity is similar in the sample and reference solutions. One of the disadvantages of the cosolvent method is that of the increased evaporation of the cosolvent-containing solution. Nevertheless, example 2 conditions have been demonstrated to work well. However, if 10 mM DMSO stock solutions are used, different DMSO stock plate dilutions need to be considered (**S --> Sd**).

As precipitation takes place to varying degrees in different pH values, the spectra of the sample solutions change in optical densities. This can be clearly seen in Fig. 10a for the sample spectra, where the sample spectra have the lowest OD values at pH 9.0 and systematically show higher OD values as pH is lowered, a pattern consistent with that of an ionizable base. The changing OD values indicate that solubility changes with pH. Fig. 10b is that of an ionizable acid, and so the pH - OD trend in the sample spectra follow the reverse order.

In the above example, the calculated reference solution concentrations, after the cosolvent had been added in step 18 (***48*** in Fig. 4a) above, are

$$C_R^{COS} = 10/\ (190+10)\ x\ 5\ /\ (145+5)\ x\ 100\ mM$$
$$= 167\ \mu M \qquad (30)$$

Note that this is nearly ten times higher than the value in the aqueous dilution method (equation 28).

The *limiting* sample concentrations (assuming no precipitation had taken place in the deep-well plate ***15*** (**Dc**) and in solution ***43*** in Fig. 4a) are calculated according to the formula

$$C_S^{COS} = 75/150\ x\ 5\ /\ (2000 + V_{KOH} - 73 + 5)\ x\ 100\ mM$$
$$= 248 - 259\ \mu M\ \text{(assuming no precipitate forms)} \qquad (31)$$

The next step requires that the spectra taken from 190 to 500 nm (in increments of 2 nm) be analyzed, to determine the area-under-the-curve (AUC) values, using equation (20) incorporating the shape-based weighting

scheme, equation (27), in the analysis. With plastic UV plates, usually only the data in the interval 240 - 500 nm are actually used in the analysis. The needed regression parameter is R^{COS}, the ratio of AUC values, equation (7).

The same considerations need to be made with regards to the limiting cases of the R^{COS} ratios, as was discussed in the aqueous dilution method.

The R = 1 situation (precipitate in both ***43*** and ***48*** in Fig. 4a) is very rarely seen in practice, since the quick handling of the reference plate and the higher amount of DMSO used in the reference plate compared to the sample plate almost always ensures that the sparingly soluble compounds are supersaturated if not dissolved. This is made more probable by the use of 3.33% v/v DMSO in the reference plate, compared to the 0.26% v/v DMSO in the sample deep well plate, and by the added cosolvent, 1-propanol at 50% v/v. In effect, there are fewer restrictions in the cosolvent method. Volatility of the cosolvent is the main disadvantage, as said before.

The solubility is calculated according to equation (8). The results for phenazopyridine stated in Table 2 were determined by the cosolvent method.

Example 3. Aqueous Solubility Determined From the Shifts in pK_a in the Presence of Non-ionizable Chemicals that Distort the Solubility-pH Profiles.

The apparent solubilities determined by equations (6) and (8) are plotted in Fig. 7 as the points *below* the horizontal lines. Those points are replotted in Fig. 8 as log S^{APP} vs. pH. The solid curves in Fig. 8

are the results of fitting the apparent solubilities to standard equations described in the Background section. From that analysis, it was possible to obtain pK_a^{APP} and S_o^{APP} values. The latter apparent intrinsic solubility constants are listed in Table 2.

As a visual check, the apparent ionization constants may be estimated as the pH values at the intersections of the horizontal and diagonal asymptotes of the solid curves.

Since the pK_as of the studied compounds are known (Table 2), it was possible to calculate the pK_a shifts (Δ values in Table 1). These shifts were used to calculate the corrected aqueous intrinsic solubilities, S_o, also listed in Table 2.

As one can see in all plots in Fig. 8, DMSO/aggregation has a significant influence on the intrinsic solubility even in 0.5% v/v DMSO. Most of the pK_as were shifted by up to one log unit. As far as we could find, this had not been recognized before in DMSO-containing solutions. Nor had a method been put forward that could correct for this effect (cf., Table 1). Table 2 summarizes the results of the corrective calculations, and compares the results to literature values. The comparison is especially good for terfenadine, probenecid, miconazole, and furosemide.

a. Chlorpromazine

The ionizable base chlorpromazine has a pK_a of 9.24 (Table 2). From the solid curve in Fig. 8, the apparent pK_a is 8.48, which is a pK_a shift of -0.76, consistent in sign with the expected behavior of bases. According to equation (11),

$$\log S_o = \log 19.4\ \mu g/mL - 0.76 = 0.528 \quad (32)$$

or

$$S_o = 10^{+0.528} = 3.4\ \mu g/mL \quad (33)$$

The horizontal asymptote in the dashed curve corresponding to chlorpromazine in Fig. 8 is 0.528 (log scale), corresponding to 3.4 μg/mL. This value is in excellent agreement with the value determined with the *p*SOL instrument, and is in reasonable agreement with the shake-flask value (Table 2). We cannot say with certainty that the pK_a shift is due solely to the effect of DMSO, or due solely to aggregation, since chlorpromazine is known to be surface-active. However, the method of our invention does not require the knowledge of the source of the effect. The method simply corrects solubilities according to the scheme in Table 1.

b. Piroxicam

The ionizable acid piroxicam (considering pH > 3) has a pK_a of 5.07 (Table 2). From the solid curve in Fig. 8, the apparent pK_a is 6.05, which is a pK_a shift of +0.98, consistent in sign with the expected behavior of acids. According to equation (10),

$$\log S_o = \log 10.5\ \mu g/mL - 0.98 = 0.041 \quad (34)$$

or

$$S_o = 10^{+0.041} = 1.1\ \mu g/mL \quad (35)$$

The horizontal asymptote in the dashed curve corresponding to piroxicam in Fig. 8 is 0.041 (log scale), corresponding to 1.1 μg/mL. This value is in poor agreement with the values determined by the shake-flask method (Table 2), 9.1 and 8-16 μg/mL. Both of the latter values were determined in the absence of DMSO. The inspection of the log S vs. pH data corresponding to the former value indicated a positive pK_a shift. Application of the correction yielded the value 3.3 μg/mL, a considerable improvement. The 8-16 μg/mL reported value did not reveal detailed log S vs. pH data. However, the authors did indicate that the pK_a was deduced from the data as 5.63. This would suggest the expected positive shift, and the corrected intrinsic solubility is thus 2.2 - 4.4 μg/mL, in very good agreement with the other 1.1 and 3.3 μg/mL values.

TABLE 2 Intrinsic Solubility, S_o, Corrected for the Drug-DMSO / Drug-Aggregate Effects

compound	pK_a	S_o^{APP} (μg/mL)	corrected S_o (μg/mL)	*p*SOL S_o (μg/mL)	Shake-Flask S_o (μg/mL)
amitriptyline	9.45 [a]	56.9	3.0	2.0 [a]	2.0 [a]
chlorpromazine	9.24 [a]	19.4	3.4	3.5 [a]	0.1 [a]
diclofenac	3.99 [b]	22.6	3.8	0.8 [b]	0.6 [b]
furosemide	10.63, 3.52 [b]	29.8	2.9	5.9 [b]	12.0 [b] (2.9 [c])
griseofulvin	non-ioniz-able	37.6	20.2		9 [d]
indomethacin	4.42 [a]	7.2	4.1	2.0 [a]	2.0 [a], 1 [e]
miconazole	6.07 [f]	11.1	1.6	0.7 [f]	
2-naphthoic acid	4.16 [f]	33.3	20.2		22.4 [g]
phenazo-pyridine	5.15 [f]	12.2	12.2	14.3 [f]	
piroxicam	5.07, 2.33 [h]	10.5	1.1		9.1 [i] (3.3 [c]), 8-16 [j] (2.2-4.4 [c])
probenecid	3.01 [f]	4.6	0.7	0.6 [f]	
terfenadine	9.53 [f]	4.4	0.1	0.1 [f]	

[a] M.A. Strafford, A. Avdeef, P. Artursson, C.A.S. Johansson, K. Luthman, C.R. Brownell, R. Lyon, *Am. Assoc. Pharm. Sci. Ann. Mtng.* 2000, poster presentation.

[b] A. Avdeef, C.M. Berger, C. Brownell, *Pharm. Res.* **2000**, *17*, 85-89.

[c] Corrected for aggregate formation: unpublished data.

[d] J. Huskonen, M. Salo, J. Taskinen, *J. Chem. Int. Comp. Soc.* **1998**, *38*, 450-456.

[e] S.H. Yalkowsky, R.-M. Dannenfelser (Eds.), AQUASOL dATAbASE of Aqueous Solubility, 5th Ed., 1998, College of Pharmacy, Univ. of Arizona, Tucson, AZ 85721.

[f] *p*ION, unpublished data.

[g] K.G. Mooney, M.A. Mintun, K.J. Himmestein, V.J. Stella, *J. Pharm. Sci.* **1981**, *70*, 13-22.

[h] A. Avdeef, K.J. Box, *Sirius Technical Application Notes, Vol. 2,* **1996**, p.110.
[i] C.R. Brownell, FDA, private correspondence, 2000.
[j] J. Jinno, D.-M. Oh, J.R. Crison, G.L. Amidon, *J. Pharm. Sci.* **2000**, *89*, 268-274 (24 hr).

c. Phenazopyridine

Phenazopyridine is an interesting molecule. It is an ionizable base, but it has *positive* pK_a shifts. The true pK_a is 5.15, but in saturated solutions, containing 0.26% v/v DMSO, the apparent pK_a is 5.83. We believe that this is a case where positively charged species either interact with DMSO or form aggregates. Being charged species, the effect is to shift the diagonal segment of the curve in a horizontal direction in the log S vs. pH plot, as indicated by the dashed line in Fig. 8. This means that the intrinsic solubility constant is not affected (Table 1).

We found a similar example in the literature for prostaglandin F2α (Table 1). The molecule is an acid, but shows a *negative* pK_a shift. The authors proposed that micelles formed to cause the shift. No effort was made to characterize the apparent pK_a. As in the case of phenazopyridine, the intrinsic solubility is not affected by the aggregation of negatively-charged species.

Example 4. Shape-based Weighting Scheme Applied to Solubility Determination

Let's consider the example in Fig. 11. Shown are the spectra of a sample and a reference solution of piroxicam. The peak maximum occurs at 359 nm. Consider the point in the spectra at 285 nm, where there is a pronounced difference in shapes between the reference spectrum and the sample spectrum. The normal error at 285 nm is calculated by equation (23) to be 0.0007 absorbance unit. For this example,

$$r_i^{REF} = a_i^{REF} / a_{max}^{REF} = 0.06/0.104 = 0.577 \quad (36)$$

$$r_i^{SAMPLE} = a_i^{SAMPLE} / a_{max}^{SAMPLE} = 0.50/1.025 = 0.488 \quad (37)$$

$$s_i = | r_i^{SAMPLE} / r_i^{REF} | = 0.488 / 0.577 = 0.944 \quad (38)$$

$$k_i = 10^{+6.3 \ (1/s - 1)} = 13 \quad (39)$$

So, the shape-biased error is a assigned to be 13 times higher in the region of shape discordance, namely, 0.008. Hence, the region near 285 nm is down-weighted by a factor of 13^2, or 169. In effect, when peak shape differences are encountered, the data are automatically taken out of consideration in the determination of the **p**-vector in the weighted linear regression analysis.

Example 5. Shape-based Weighting Scheme Applied to Permeability Determination

The shape-based weighting scheme we developed is general and can be applied to any spectroscopic determination of concentrations using multi-wavelength data. We have applied the UV-based shape analysis weighting scheme to the determination of permeability constants. This scheme is also applicable to any concentration determination using spectral data.

Example 6. Improved Aqueous Universal Buffer Formulation Possessing Low-UV Components and Being Free of Phosphate and Citrate

In this example and in Example 7 below, two new improved universal buffers are described for high-throughput solubility applications, *and other applications where concentration measurements are made at controlled pH using spectrophotometric methods, including permeability measurements.* We sought to discover an aqueous and a cosolvent-water universal buffer which would make solubility measurements more reliable. We wanted buffers that would not interact with the molecules being studied, which meant that the background salt concentrations needed to be low. For this reason, we had to stay away from *phosphate* as a buffer component due to its strong tendency to cause salts of positively-charged drug substances to precipitate [W.H. Streng, S.K. Hsi, P.E. Helms, H.G.H. Tan, *J. Pharm. Sci.* **1984**, *73*, 1679-1684]. We wanted to avoid ion-pair interactions between buffer components and the sample, so we wanted to stay away from lipophilic buffers. Since we wanted our detection

system to obtain as much signal due to sample and as little due to background buffers, we wanted the UV absorption due to the buffer to be as low as possible. Citric acid buffer component would not have been a good choice from this consideration. We wanted the pH vs. volume of alkaline titrant relationship to be as linear as possible, so that we could easily predict how much of a pH change a given sample may impart on the buffer system.

None of the commonly known universal buffers [D.D. Perrin, B. Dempsey, *Buffers for pH and Metal Ion Control*, Chapman and Hall, London, 1974; A. Avdeef, J.J. Bucher, *Anal. Chem.* **1978**, *50*, 2137-2142] fit the desired profile. We found that the zwitterionic Good's buffers [Good, Winget, Winter, Connolly, Izawa, Singh, *Biochemistry* **1966**, *5*, 467] best fit our desired characteristics. Several buffers were tested as possible candidates, but one set was discovered to be outstanding.

The best candidates for the aqueous universal buffer were discovered to be the combination: acetic acid, MES(4-morpholine-ethanesulfonic acid), HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and boric acid. MES and boric acid had virtually no UV absorption above 210 nm. HEPES and acetic acid had pK_as in the right positions to make them attractive choices, even though their UV absorptivities were not the lowest.

Each component was initially chosen to be 5 mM, to form an equimolar mixture, but better ratios were discovered afterwards.

Table 3 Composition of the Aqueous Universal Buffer[a]

Buffer Component	*MW*	*Weight (g) Used to Make 1 L*	*Concen-tration (mM)*	*pK_a (I=0.020M[b])*
Sodium Acetate, Trihydrate	136.08	0.7626	5.60	4.64
MES monohydrate	213.26	1.0286	4.82	6.08
HEPES	238.31	1.1915	5.00	7.48, 3.01
Boric Acid	61.83	0.4083	6.60	9.13

[a] To achieve a starting pH 3.0, 16.26 mL of 0.5M HCl were added per liter, corresponding to 0.008 M added HCl. [b] I = average ionic strength in the titration from pH 3 - 10.

Table 3 shows the best component concentrations, along with other properties of the components. Obviously, other forms of the buffering materials (e.g., sodium acetate, anhydrous) could be utilized. Figure 12 shows the pH-volume curve, where 2.00 mL of aqueous universal buffer solution were titrated with 0.5 M KOH. The linear regression of pH vs. titrant volume produced the equation: pH = 3.10 + 72.08 V_{KOH}, with the *extraordinary* standard deviation of 0.02 pH units.

Example 7. Improved Aqueous-Cosolvent Universal Buffer Formulation Possessing Low-UV Components and Being Free of Phosphate and Citrate

Finding suitable candidates for a cosolvent universal buffer solution was problematic, since cosolvents change the pK_as of the buffer components, and it is difficult to predict accurately to what extent the pK_as would be different. The best candidates for the cosolvent buffer (Table 4), where the cosolvent acetonitrile was mixed with water in 1:1 proportion were discovered to be glycolic acid, MES, HEPES, and taurine (2-aminoethanesulfonic acid).

Figure 13 shows the pH-volume curve, where 2.00 mL of acetonitrile-water universal buffer solution were titrated with 0.5 M KOH. The linear regression of pH vs. titrant volume produced the equation: $pH = 3.01 + 75.59\ V_{KOH}$, with a good standard deviation of 0.06 pH units.

Table 4 Composition of the 1:1 Acetonitrile-Water Universal Buffer[a]

Buffer Component	*MW*	*Weight (g) Used to Make 1 L*	*Concentration (mM)*	*pK_a (I=0.012M, 50 vol% CH_3CN)*
Glycolic Acid	76.05	0.4000	5.26	4.56
MES monohydrate	213.26	1.0000	4.69	6.24
HEPES	238.31	1.1915	5.00	7.54, 2.91
Taurine	125.15	0.7000	5.60	8.95

[a] To achieve a starting pH 3.0, 6.76 mL of 0.5M HCl were added per liter, corresponding to 0.003366 M added HCl. Initially, 500 mL acetonitrile was added to the weighed components in a 1 L volumetric flask, to which were added 6.76 mL 0.5 M HCl. Distilled water was then added to make 1.00 L solution.

Variations of the above methods and analytical devices will be apparent to those with ordinary skill in the field.

CLAIMS

We claim:

1. A method for determining the solubility of a compound, said compound having limited solubility in water and in a buffer, comprising:

a. preparing a saturated solution of said compound, after which any insoluble compound is filtered off,

b. preparing a reference solution of said compound, under conditions avoiding or suppressing precipitation,

c. measuring a spectrophotometric property of said saturated and reference solutions and said buffer, wherein said buffer serves as a blank, and

d. analyzing said spectrophotometric property to determine the solubility of said compound.

2. The method of claim 1, wherein said spectrophotometric property is selected from the group consisting of UV range spectrophotometry, visible range spectrophotometry, colorimetry, light scattering detection, polarimetry, optical rotation, fluorimetry and circular dichroism detection.

3. The method of claim 1 wherein:

a. said reference solution is prepared by dissolving a known quantity of said compound or stock solution containing said compound in buffer to produce a solution free of precipitate, and

b. said saturated solution is prepared by dissolving a known quantity of said compound or stock solution containing said compound in buffer to produce a solution with precipitate dispersed therein.

4. The method of claim 1 wherein said compound is presented as a stock solution in DMSO and said spectrophotometric property is UV range absorbance.

5. The method of claim 4 wherein:

a. said reference solution is prepared by diluting said stock solution with DMSO and buffer, and

b. said saturated solution is prepared by diluting said stock solution with buffer, after which any insoluble compound is filtered off.

6. The method of claim 5, wherein:

a. said reference solution is prepared by diluting one aliquot of said stock solution with 19 aliquots of DMSO; and one aliquot of said diluted solution is further diluted with 3 aliquots of DMSO and 400 aliquots of buffer,

b. said saturated solution is prepared by diluting 1 aliquot of said stock solution with 400 aliquots of buffer, allowing said solution to equilibrate for several hours, after which any insoluble compound is filtered off, and

c. said analysis requires that (1) the absorbance of said reference solution be compared to (2) the absorbance of said saturated solution at one or more wavelengths, wherein, all of said solutions are adjusted to the same pH.

7. The method of claim 4, wherein

a. said blank is prepared by diluting an aqueous buffer with cosolvent,

b. said reference solution is prepared by diluting stock solution with DMSO, buffer, and cosolvent,

c. said saturated solution is prepared by diluting stock solution with buffer, filtering off any insoluble compound, and further diluting said solution with cosolvent.

8. The method of claim 7 wherein said cosolvent is selected from the group consisting of acetonitrile, methanol, ethanol, iso-propanol, 1-propanol, 1,4-dioxane, dimethylformamide, acetone, ethylene glycol, propylene glycol, polyethylene glycol 400, tetrahydrofuran, DMSO and mixtures thereof.

9. The method of claim 8, wherein said cosolvent is 1-propanol.

10. The method of claim 9, wherein:

a. said blank is prepared by diluting 1 aliquot of said buffer with 1 aliquot of a cosolvent,

b. said reference solution is prepared by diluting 1 aliquot of stock solution with 19 aliquots of DMSO; and 1 aliquot of said diluted solution is further diluted by 15 aliquots of said buffer and 15 aliquots of cosolvent;

c. said saturated solution is prepared by diluting 1 aliquot of stock solution with 400 aliquots

of buffer, allowing said solution to equilibrate for several hours, after which any insoluble compound is filtered off; and 1 aliquot of said resulting solution is further diluted by 1 aliquot of cosolvent;

d. said analysis requires that (1) the absorbance of said reference solution be compared to (2) the absorbance of said saturated solution at one or more wavelengths, wherein, all of said solutions are adjusted to the same pH.

11. The method of claim 1, wherein

a. said solubility is intrinsic solubility,

b. said compound is ionizable and anomalies are present in solution, and wherein said analysis requires that the log of solubility of said saturated solution be plotted against pH to produce a solubility-pH curve,

c. the displacement of the pK_a caused by said anomaly can be determined by independently determining the pK_a of said compound, and

d. said displacement of pK_a can be used to removing the effect of the anomaly and determine intrinsic solubility of said compound.

12. The method of claim 11, wherein said anomaly is caused by one or more factors selected from the group consisting of:

a. self-association effects,

b. presence of bile acids or other surfactants,

c. presence of cyclodextrins,

d. presence of ion-pair forming counterions,

e. presence of non-ionizable polymers,

f. presence of phospholipids, and

g. presence of DMSO.

13. The method of claim 12, wherein said anomaly is caused by the presence of DMSO, wherein:

a. said pK_a of said compound in buffer free of anomaly can be determined by using known analytical techniques,

b. the pK_a of said compound in said solution having said anomaly is obtained by determining the pH of the intersection of horizontal and diagonal asymptotes of said solubility-pH curve,

c. the difference between (1) the pK_a of said compound in buffer free of anomaly and (2) the pK_a of said compound in said solution is determined, and

d. said difference is added to the log of the apparent intrinsic solubility to determine the log of the intrinsic solubility of said compound free of anomaly,

provided that, (1) for ionizable acids, pK_a of said compound in said solution minus the pK_a of said compound in buffer free of anomaly is greater than zero, or (2) for ionizable bases, pK_a of said compound in said solution minus the pK_a of said compound in buffer free of anomaly is less than zero,

14. The method of claim 1 wherein said spectrophotometric property is determined at more than one point, and the values at said points are corrected to account for the presence of interferences, said corrections being based on

a. comparing the shape of the spectrophotometric property curve of said reference solution to the shape of a curve of said compound in saturated solution, and

b. applying weighting factors, wherein those portions of the curve of said reference solution that differ from the corresponding curve prepared in saturated solution contribute less to said method than those where said shape is similar to said curve relating to said saturated solution.

15. The method of claim 14 wherein said spectrophotometric property is UV absorbance.

16. The method of claim 15 applied to determination of concentrations of sample solutions when comparing UV absorbance spectra of sample solutions to UV absorbance spectra of reference solutions.

17. The method of claim 1, wherein said buffer provides buffering capacity over the range of pH 3-10 and has low-UV absorbance.

18. The method of claim 17 wherein said buffer comprises acetic acid, MES, HEPES and boric acid.

19. The method of claim 17, wherein said buffer comprises glycolic acid, MES, HEPES, taurine, and acetonitrile.

20. An instrument for determining the solubility of a compound that is sparingly soluble in water and in a buffer, said instrument comprising means for:

a. accurately titrating components of a solution,

b. collecting components of a solution,

c. mixing solutions,

d. filtering insoluble residues from solutions,

e. adjusting pH values of solutions,

f. determining a spectrophotometric property of a solution, and

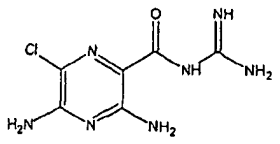
g. performing calculations, including those relating to said UV absorbance spectra.

21. The instrument of claim 20 for determining the solubility of a compound that is sparingly soluble in water and in a buffer, wherein said means for determining a spectrophotometric property of a solution is selected from the group consisting of a UV range spectrophotometer, a visible range spectrophotometer, a colorimeter, a light scattering detector, a polarimeter, an optical rotation, a fluorimeter, or a circular dichroism detector.

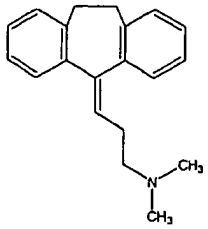
22. A buffer providing buffering capacity over the range of pH 3-10 and having low-UV absorbance.

23. The buffer of claim 22 comprising sodium acetate, MES monohydrate, HEPES and boric acid.

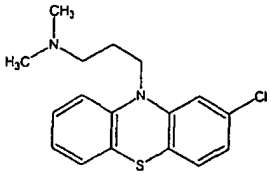
24. The buffer of claim 22 comprising glycolic acid, MES monohydrate, HEPES, taurine, and acetonitrile.



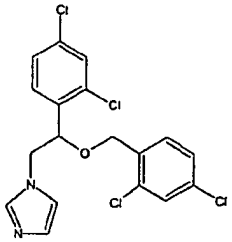
amiloride



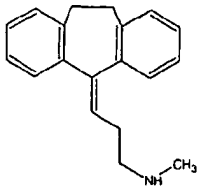
amitriptyline



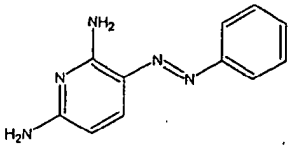
chlorpromazine



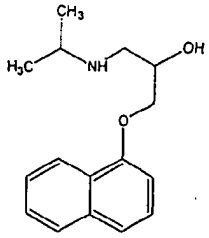
miconazole



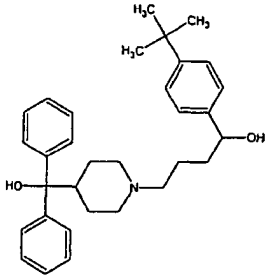
nortriptyline



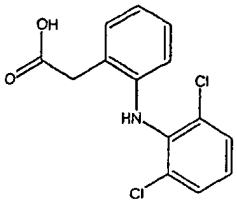
phenazopyridine



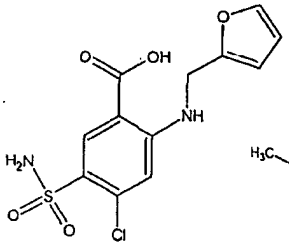
propranolol



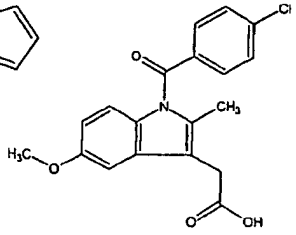
terfenadine



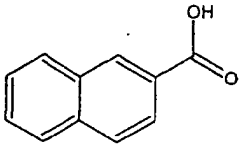
diclofenac



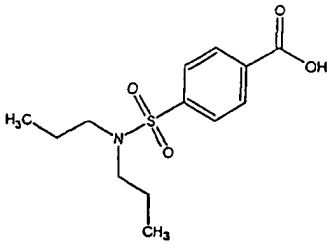
furosemide



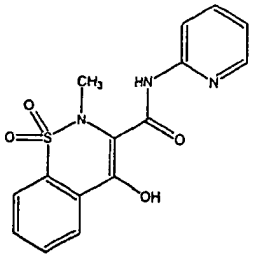
indomethacin



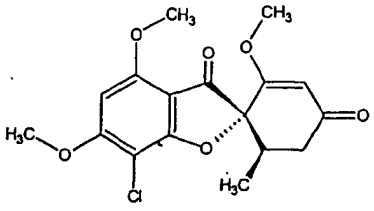
2-naphthoic acid



probenecid



piroxicam



griseofulvin

FIG. 1

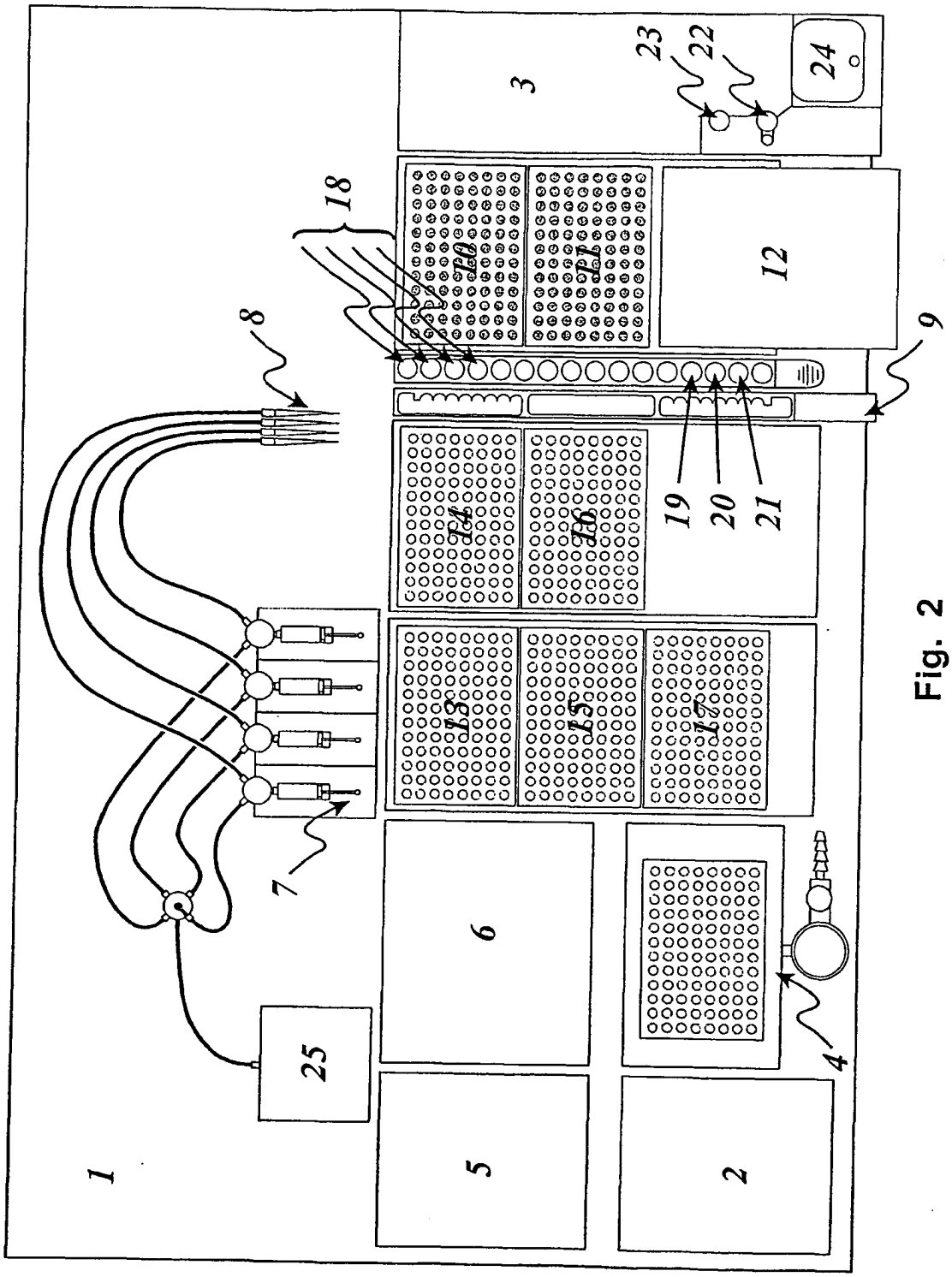


Fig. 2

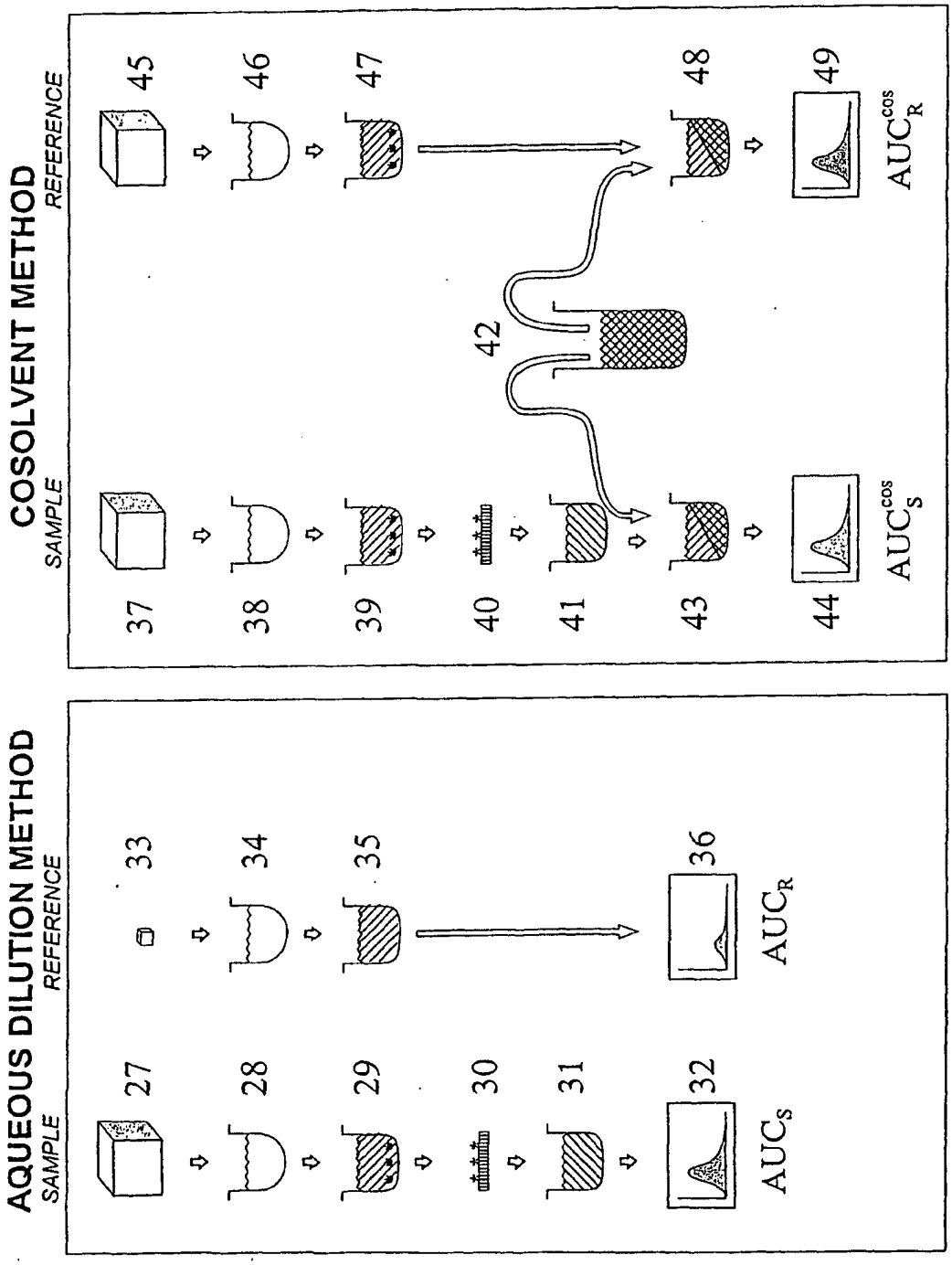

AQUEOUS DILUTION METHOD
SAMPLE
REFERENCE
27
28
29
30
31
32
33
34
35
36
AUC$_S$
AUC$_R$
COSOLVENT METHOD
SAMPLE
REFERENCE
37
38
39
40
41
42
43
44
45
46
47
48
49
AUC$_S^{cos}$
AUC$_R^{cos}$

FIG. 3a

FIG. 4a

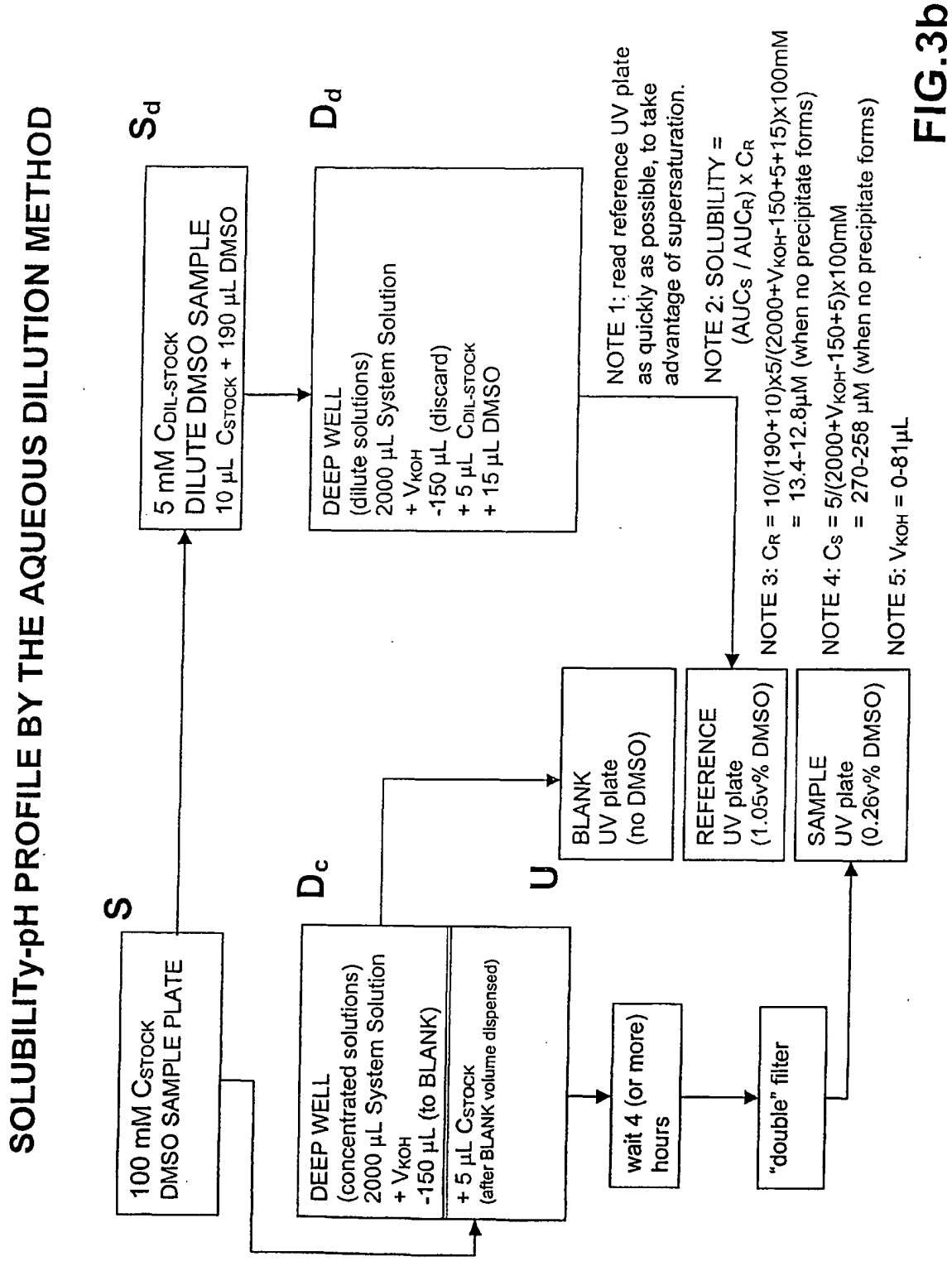

SOLUBILITy-pH PROFILE BY THE AQUEOUS DILUTION METHOD
S
100 mM CSTOCK
DMSO SAMPLE PLATE
Sd
5 mM CDIL-STOCK
DILUTE DMSO SAMPLE
10 µL CSTOCK + 190 µL DMSO
Dd
DEEP WELL
(dilute solutions)
2000 µL System Solution
+ VKOH
-150 µL (discard)
+ 5 µL CDIL-STOCK
+ 15 µL DMSO
Dc
DEEP WELL
(concentrated solutions)
2000 µL System Solution
+ VKOH
-150 µL (to BLANK)
+ 5 µL CSTOCK
(after BLANK volume dispensed)
U
BLANK
UV plate
(no DMSO)
wait 4 (or more)
hours
"double" filter
REFERENCE
UV plate
(1.05v% DMSO)
SAMPLE
UV plate
(0.26v% DMSO)
NOTE 1: read reference UV plate as quickly as possible, to take advantage of supersaturation.
NOTE 2: SOLUBILITY = (AUCS / AUCR) x CR
NOTE 3: CR = 10/(190+10)x5/(2000+VKOH-150+5+15)x100mM
= 13.4-12.8µM (when no precipitate forms)
NOTE 4: CS = 5/(2000+VKOH-150+5)x100mM
= 270-258 µM (when no precipitate forms)
NOTE 5: VKOH = 0-81µL

FIG.3b

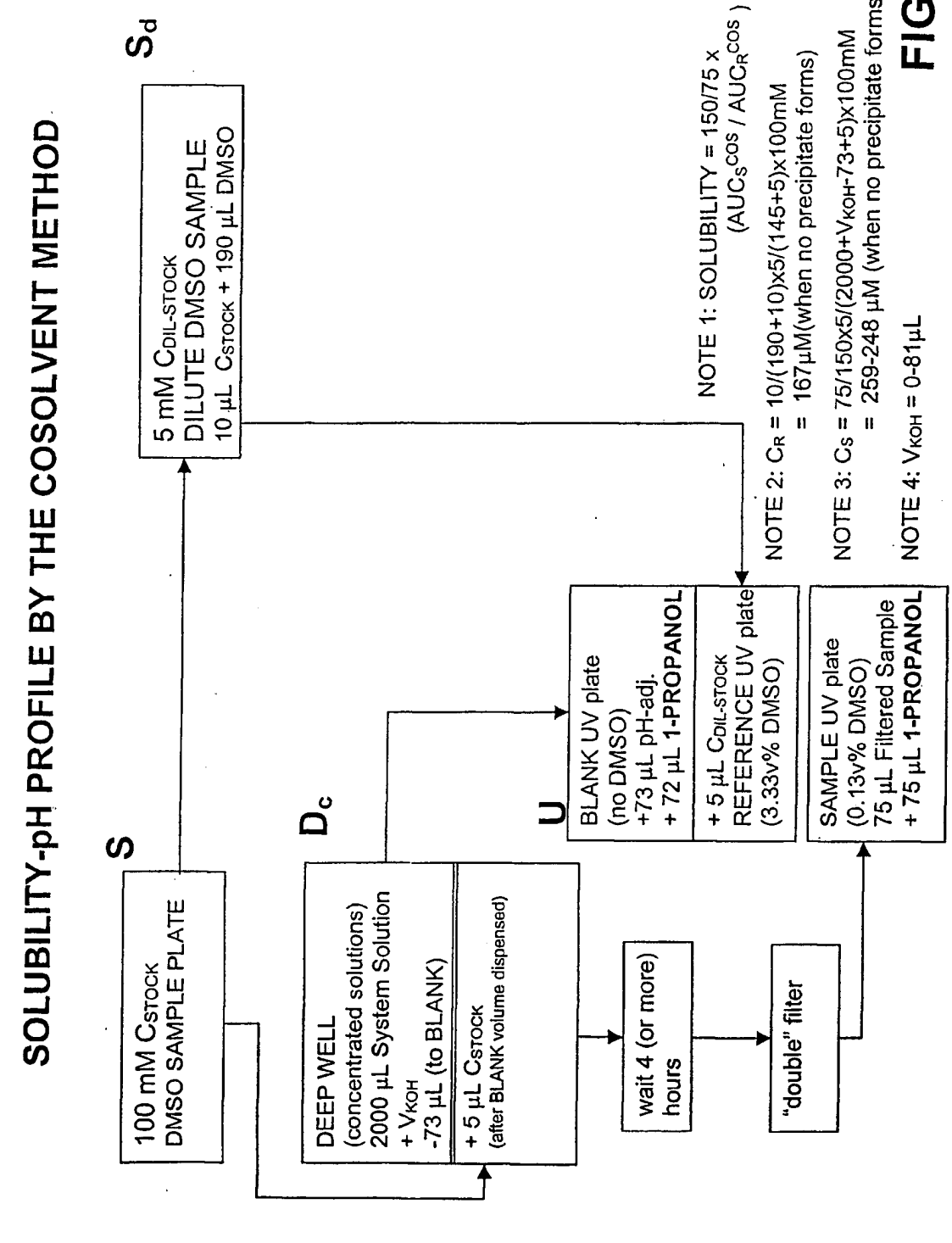

SOLUBILITY-pH PROFILE BY THE COSOLVENT METHOD
S
100 mM CSTOCK
DMSO SAMPLE PLATE
Sd
5 mM CDIL-STOCK
DILUTE DMSO SAMPLE
10 µL CSTOCK + 190 µL DMSO
Dc
DEEP WELL
(concentrated solutions)
2000 µL System Solution
+ VKOH
-73 µL (to BLANK)
+ 5 µL CSTOCK
(after BLANK volume dispensed)
wait 4 (or more) hours
"double" filter
U
BLANK UV plate
(no DMSO)
+73 µL pH-adj.
+ 72 µL 1-PROPANOL
+ 5 µL CDIL-STOCK
REFERENCE UV plate
(3.33v% DMSO)
SAMPLE UV plate
(0.13v% DMSO)
75 µL Filtered Sample
+ 75 µL 1-PROPANOL
NOTE 1: SOLUBILITY = 150/75 x (AUCSCOS / AUCRCOS) x CR
NOTE 2: CR = 10/(190+10)x5/(145+5)x100mM
= 167µM(when no precipitate forms)
NOTE 3: CS = 75/150x5/(2000+VKOH-73+5)x100mM
= 259-248 µM (when no precipitate forms)
NOTE 4: VKOH = 0-81µL

FIG. 4b

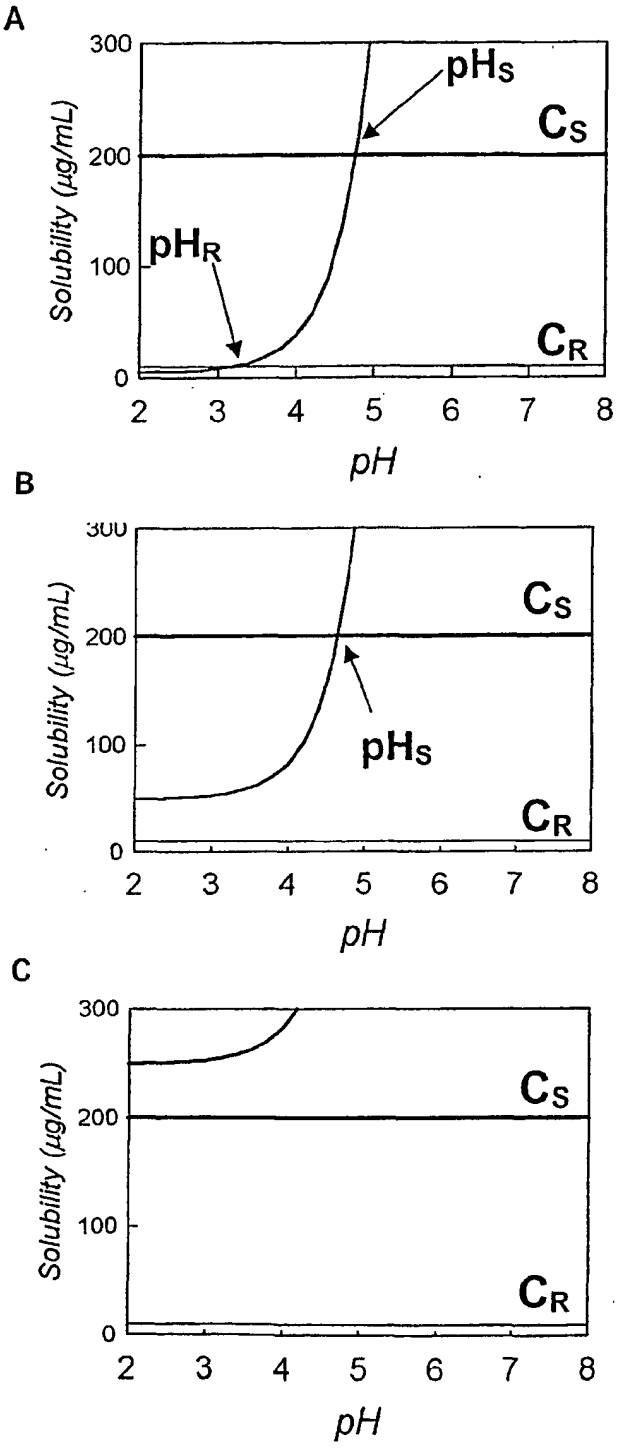

A
300
200
100
0
Solubility (µg/mL)
pHS
CS
pHR
CR
2 3 4 5 6 7 8
pH
B
300
200
100
0
Solubility (µg/mL)
CS
pHS
CR
2 3 4 5 6 7 8
pH
C
300
200
100
0
Solubility (µg/mL)
CS
CR
2 3 4 5 6 7 8
pH

FIG. 5

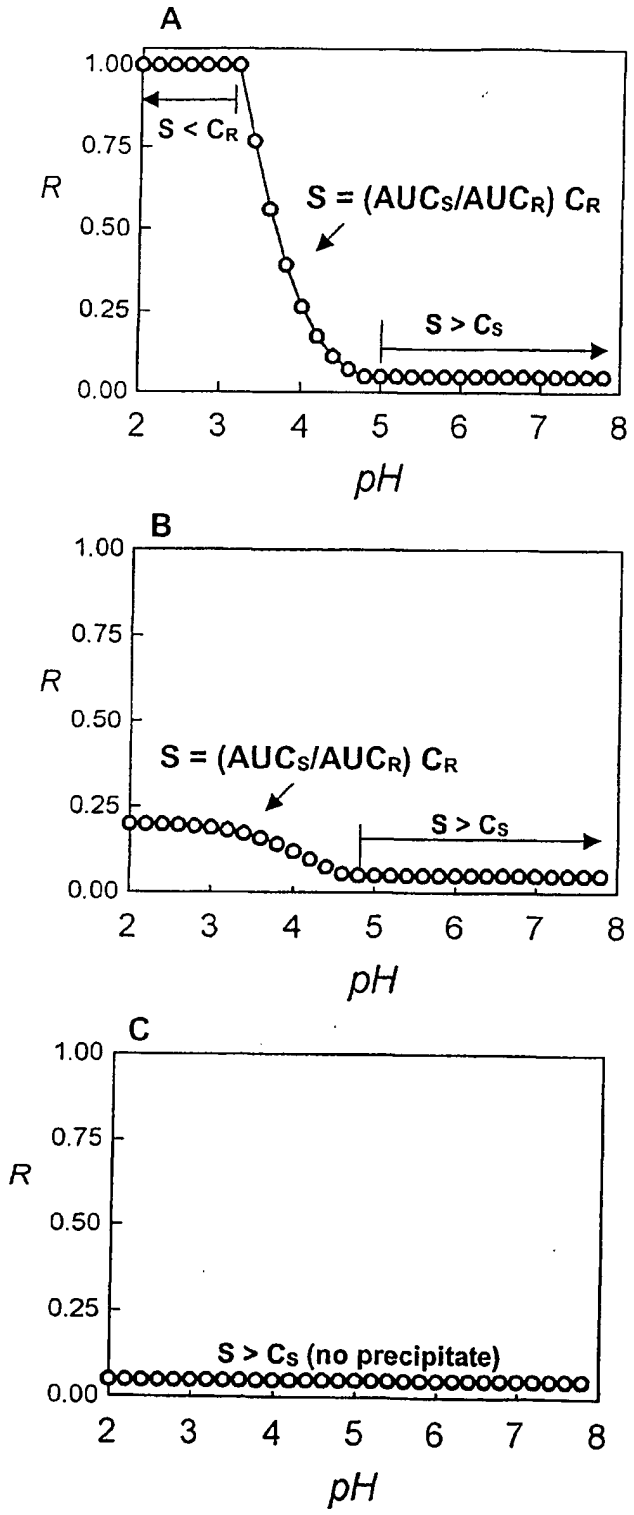

A
1.00
0.75
R
0.50
0.25
0.00
S < CR
S = (AUCS/AUCR) CR
S > CS
2 3 4 5 6 7 8
pH
B
1.00
0.75
R
0.50
0.25
0.00
S = (AUCS/AUCR) CR
S > CS
2 3 4 5 6 7 8
pH
C
1.00
0.75
R
0.50
0.25
0.00
S > CS (no precipitate)
2 3 4 5 6 7 8
pH

FIG. 6

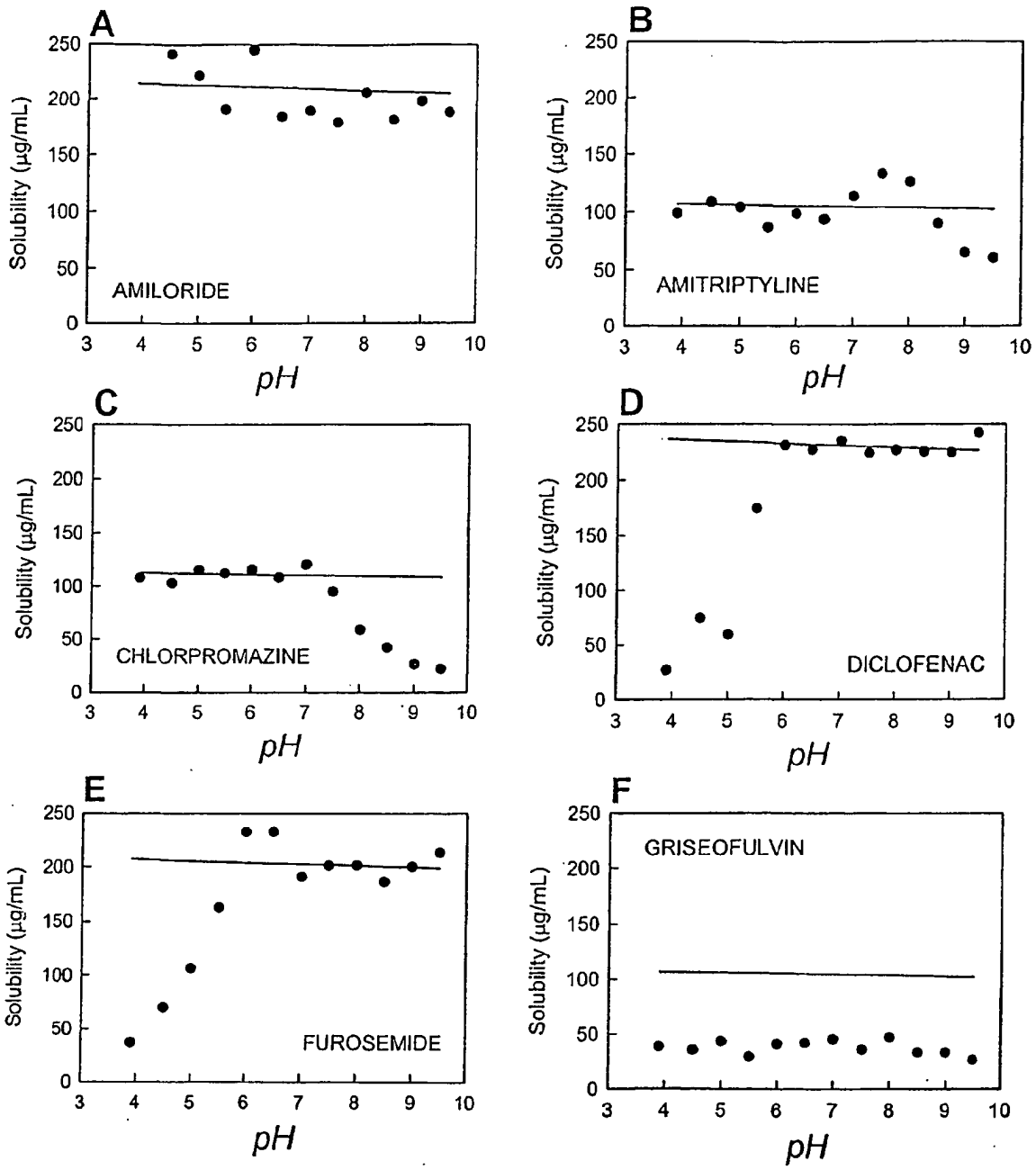

A
AMILORIDE
B
AMITRIPTYLINE
C
CHLORPROMAZINE
D
DICLOFENAC
E
FUROSEMIDE
F
GRISEOFULVIN
Solubility (µg/mL)
pH

FIG. 7

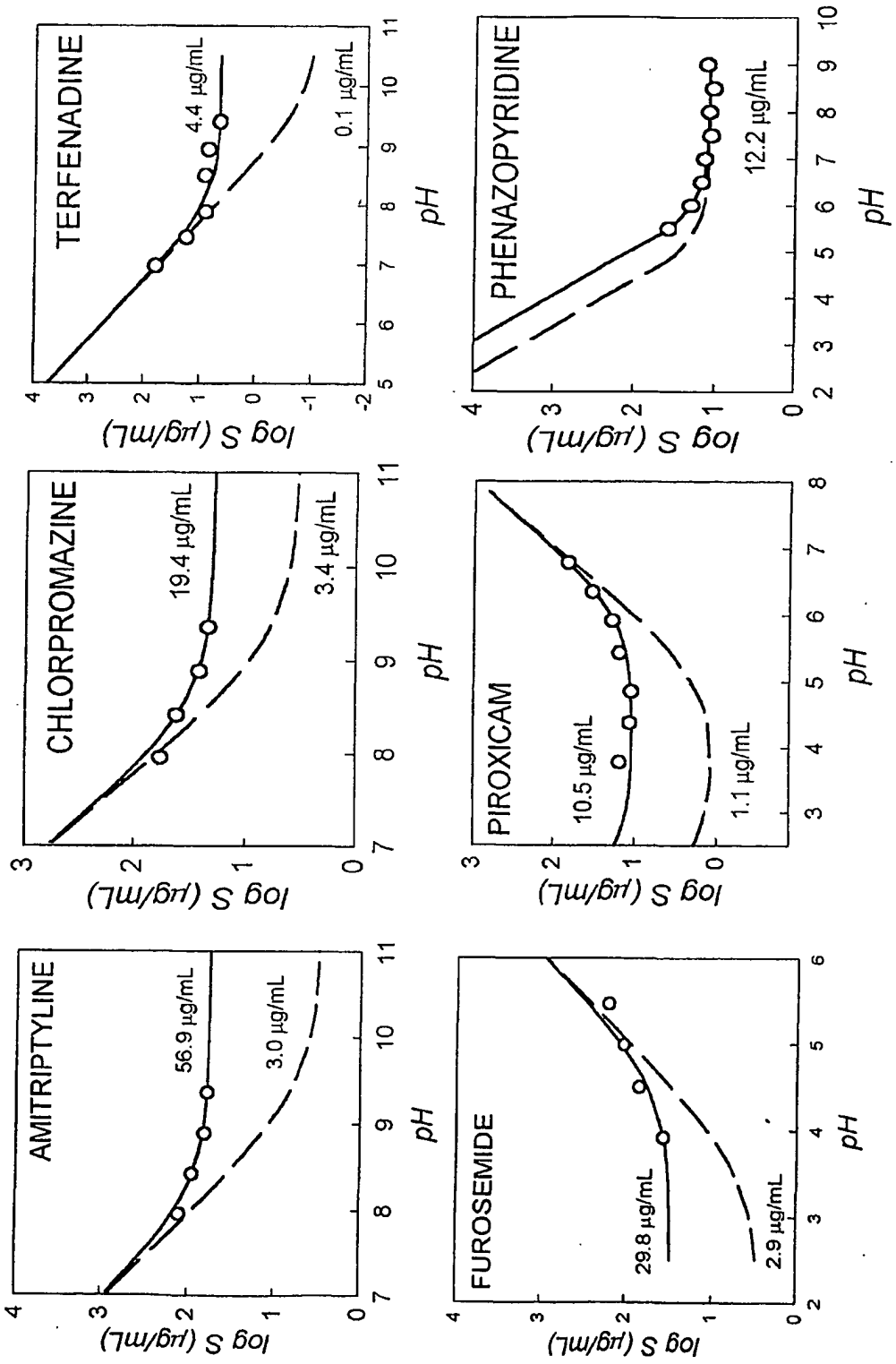

TERFENADINE
4.4 µg/mL
0.1 µg/mL
log S (µg/mL)
pH
PHENAZOPYRIDINE
12.2 µg/mL
log S (µg/mL)
pH
CHLORPROMAZINE
19.4 µg/mL
3.4 µg/mL
log S (µg/mL)
pH
PIROXICAM
10.5 µg/mL
1.1 µg/mL
log S (µg/mL)
pH
AMITRIPTYLINE
56.9 µg/mL
3.0 µg/mL
log S (µg/mL)
pH
FUROSEMIDE
29.8 µg/mL
2.9 µg/mL
log S (µg/mL)
pH

FIG.8

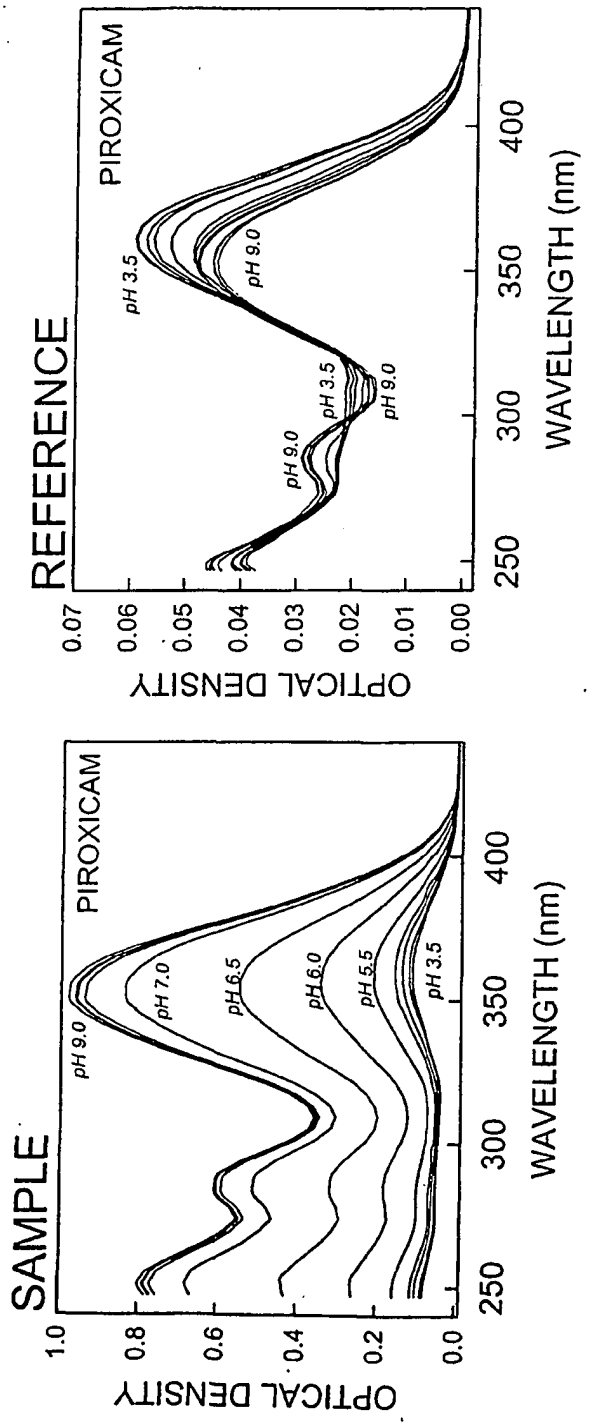

REFERENCE
PIROXICAM
pH 3.5
pH 9.0
pH 3.5
pH 9.0
pH 9.0
pH 3.5
OPTICAL DENSITY
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
250
300
350
400
WAVELENGTH (nm)
SAMPLE
PIROXICAM
pH 9.0
pH 7.0
pH 6.5
pH 6.0
pH 5.5
pH 3.5
OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0.0
250
300
350
400
WAVELENGTH (nm)

FIG. 9

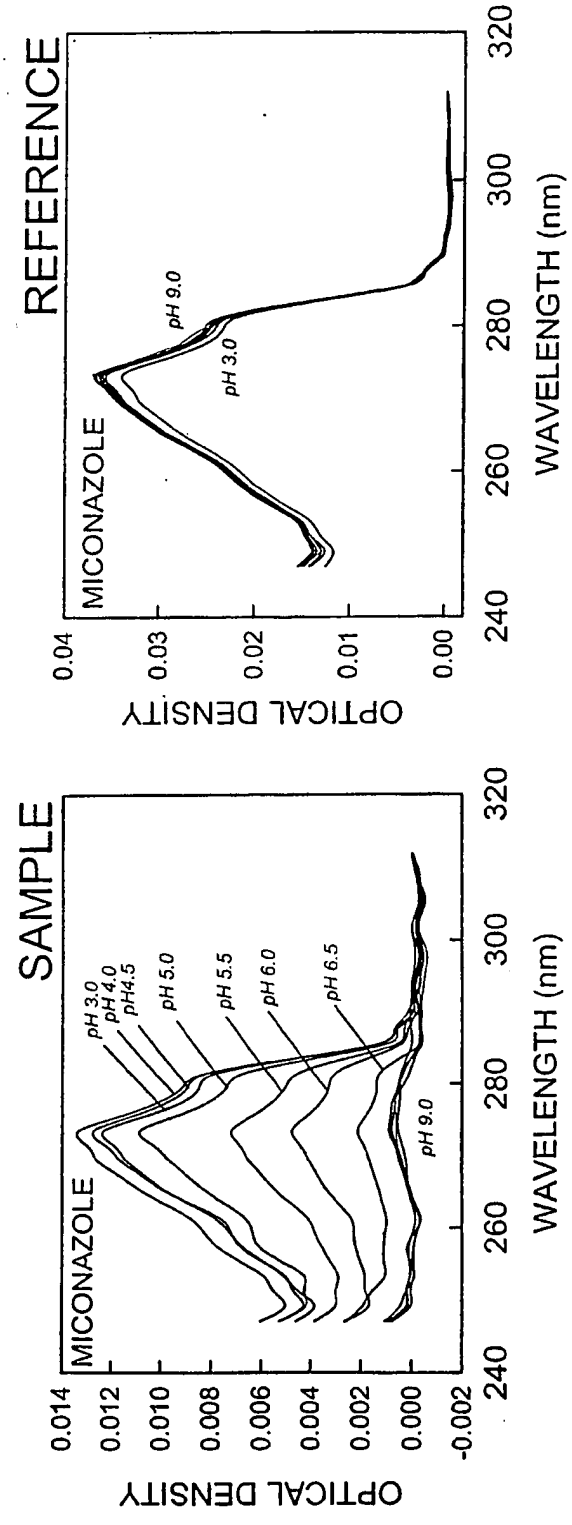

REFERENCE
MICONAZOLE
pH 9.0
pH 3.0
OPTICAL DENSITY
0.04
0.03
0.02
0.01
0.00
240
260
280
300
320
WAVELENGTH (nm)
SAMPLE
MICONAZOLE
pH 3.0
pH4.0
pH4.5
pH 5.0
pH 5.5
pH 6.0
pH 6.5
pH 9.0
OPTICAL DENSITY
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
-0.002
240
260
280
300
320
WAVELENGTH (nm)

FIG. 10a

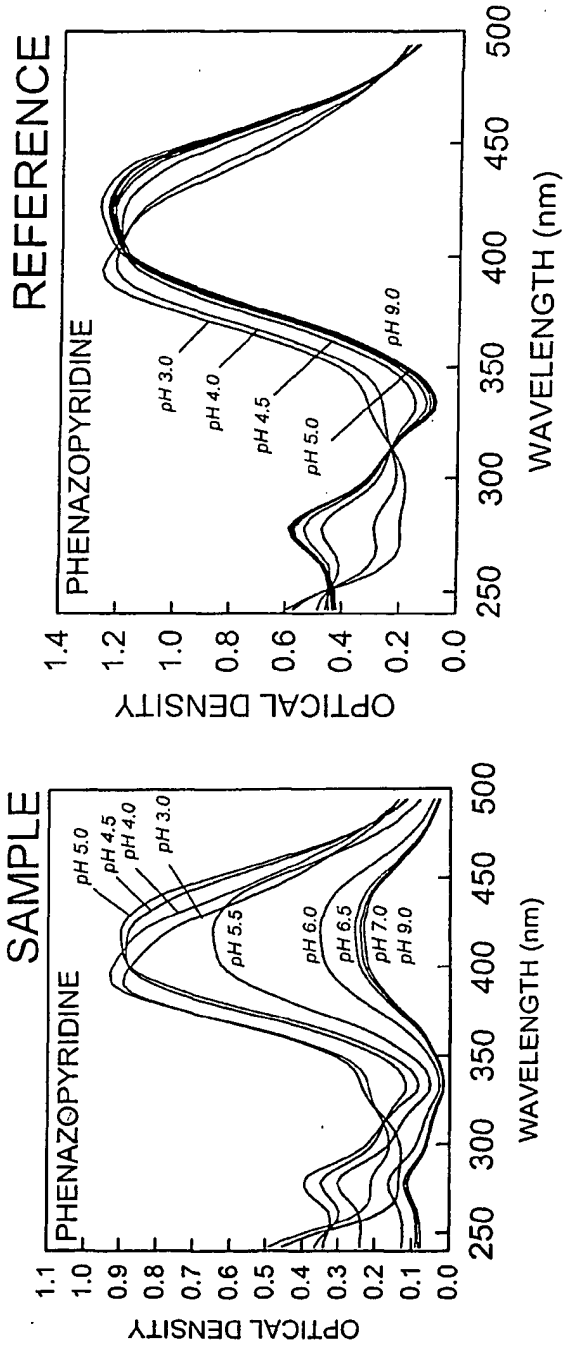

REFERENCE
PHENAZOPYRIDINE
pH 3.0
pH 4.0
pH 4.5
pH 5.0
pH 9.0
OPTICAL DENSITY
WAVELENGTH (nm)
SAMPLE
PHENAZOPYRIDINE
pH 5.0
pH 4.5
pH 4.0
pH 3.0
pH 5.5
pH 6.0
pH 6.5
pH 7.0
pH 9.0
OPTICAL DENSITY
WAVELENGTH (nm)

FIG. 10b

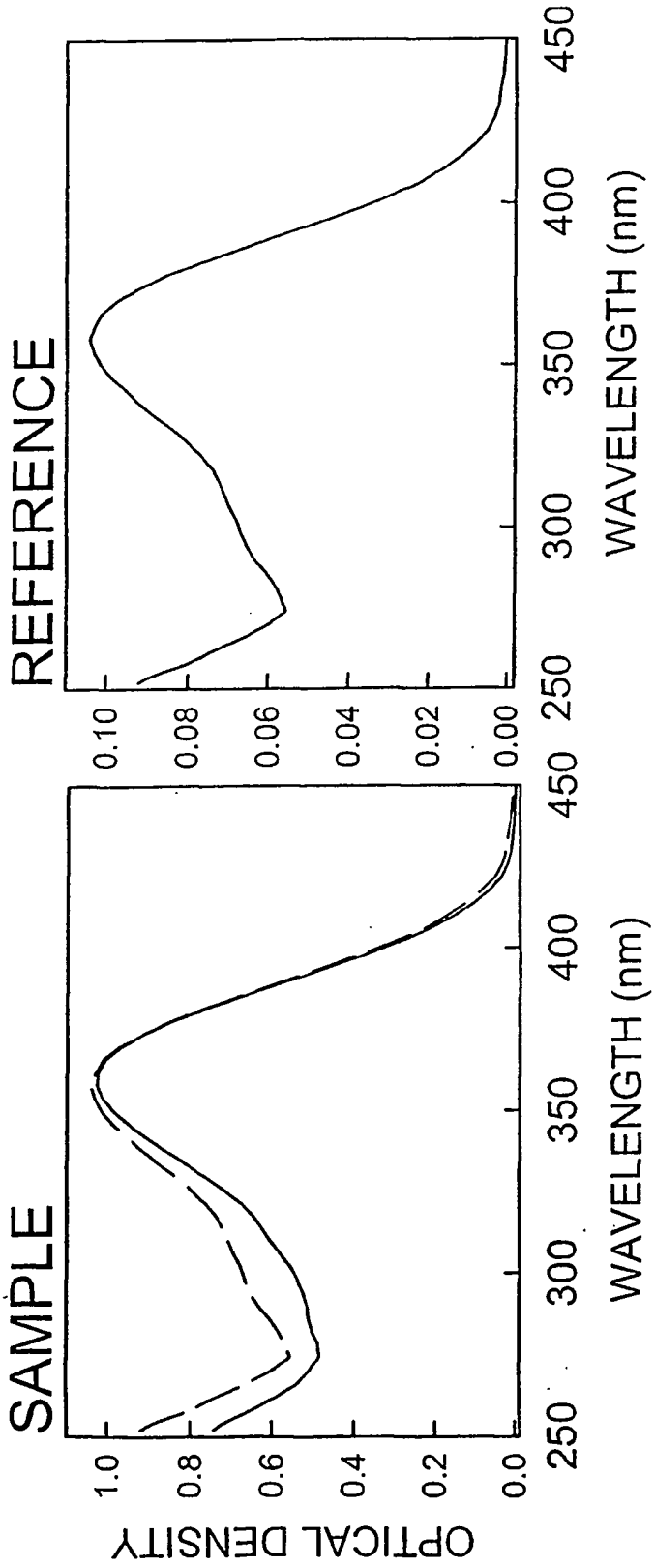

SAMPLE
REFERENCE
OPTICAL DENSITY
WAVELENGTH (nm)
WAVELENGTH (nm)
1.0
0.8
0.6
0.4
0.2
0.0
0.10
0.08
0.06
0.04
0.02
0.00
250
300
350
400
450

FIG. 11

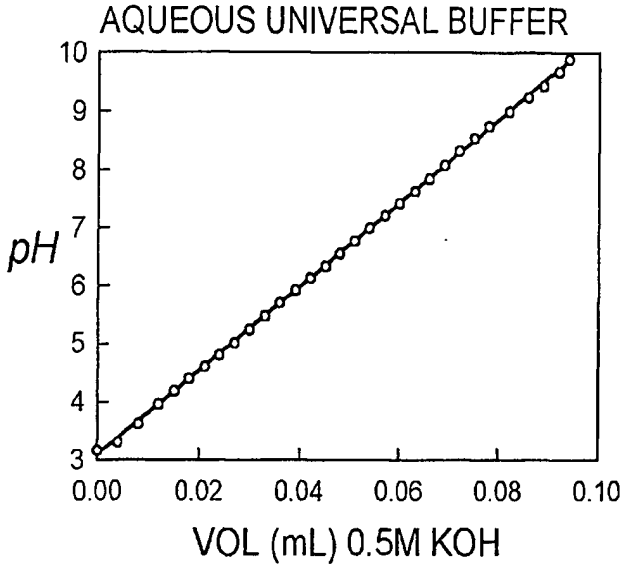

AQUEOUS UNIVERSAL BUFFER
pH
10
9
8
7
6
5
4
3
0.00
0.02
0.04
0.06
0.08
0.10
VOL (mL) 0.5M KOH

FIG. 12

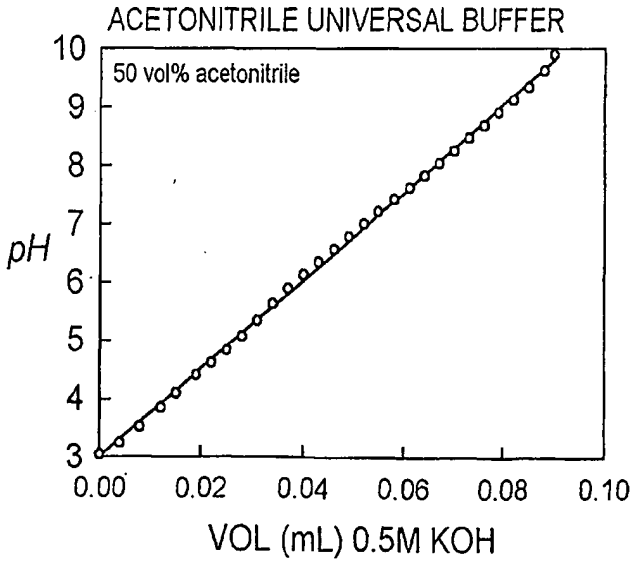

ACETONITRILE UNIVERSAL BUFFER
50 vol% acetonitrile
pH
10
9
8
7
6
5
4
3
0.00
0.02
0.04
0.06
0.08
0.10
VOL (mL) 0.5M KOH

FIG. 13

INTERNATIONAL SEARCH REPORT

Application No
PCT/US 01/02377

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N21/33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, INSPEC, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIPINSKI C A ET AL: "EXPERIMENTAL AND COMPUTATIONAL APPROACHES TO ESTIMATE SOLUBILITY AND PERMEABILITY IN DRUG DISCOVERY AND DEVELOPMENT SETTINGS" ADVANCED DRUG DELIVERY REVIEWS,AMSTERDAM,NL, vol. 23, no. 1/03, 1997, pages 3-25, XP000892390 ISSN: 0169-409X cited in the application page 15, right-hand column, paragraph 2 -page 17, left-hand column, paragraph 3 ---	1,2,4
A	WO 99 13328 A (BEVAN CHRISTOPHER DAVID ;GLAXO GROUP LTD (GB); HILL ALAN PETER (GB) 18 March 1999 (1999-03-18) cited in the application claims 1,8 --- -/--	1,2,20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 June 2001	22/06/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Krametz, E

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int Application No
PCT/US 01/02377

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 906 580 A (MESEROLE FRANK B) 6 March 1990 (1990-03-06) column 2, line 12 - line 29 claim 1 -----	1,2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int al Application No
PCT/US 01/02377

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9913328 A	18-03-1999	AU 9083498 A	29-03-1999
		CN 1278331 T	27-12-2000
		EP 1012599 A	28-06-2000
US 4906580 A	06-03-1990	AU 5102090 A	24-08-1990
		EP 0455734 A	13-11-1991
		WO 9008950 A	09-08-1990
		US 4939153 A	03-07-1990